ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF MINOCYCLINE HYDROCHLORIDE IN BULK AND TABLET DOSAGE FORMS USING RP-HPLC METHOD

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1. INTRODUCTION

The Objectives of the present investigation:

The main objectives of this investigation is to develop and validate a RP-HPLC-PDA method for the estimation of Minocycline hydrochloride in bulk, pharmaceutical dosage forms compatible for LC-MS Methods, which is rapid, sensitive and economical.

A. Method development

i) Optimization of LC conditions

B. Method Validation

i) Specificity

ii) Linearity

iii) Precision

iv) Accuracy

v) Robustness

vi) LOD and LOQ

vii) System Suitability

viii) Stability of solutions and

ix) Assay

HIGH PRESSURE LIQUID CHROMATOGRAPHY (HPLC)

High performance liquid chromatography is basically a highly improved form of column chromatography. It is the most widely used form of chromatography. Instead of a solvent being allowed to drip through a column under gravity, it is forced through the column under high pressures and this improves separation. The

improvements of HPLC have enabled liquid chromatography to match the great success of gas chromatography. The separation principles involved may include[15]

1) Adsorption
2) Partition
3) Ion exchange
4) Gel permeation
5) Affinity

HPLC can be used for both qualitative and quantitative analysis. In qualitative analysis, the retention time of the compounds are made use of. In quantitative analysis on the other hand, the area under the peak, which is proportional to the concentration of the compound is used.

The HPLC is the method of choice in the field of analytical chemistry, since this method is specific, robust, linear, precise and accurate and the limit of detection is low and also it offers the following advantages[16]

- Speed (many analysis can be accomplished in 20 min or less)
- Greater sensitivity (various detectors can be employed)
- Improved resolution (wide variety of stationary phases)
- Reusable columns (expensive columns but can be used for many analysis)
- Ideal for the substances of low viscosity
- Easy sample recovery, handling and maintenance.
- Instrumentation leads itself to automation and quantification (less time and less labour), Precise and reproducible
- Integrator itself does calculations.

HPLC Instrumentation

An HPLC apparatus consists of a pumping system, an injector, a chromatographic column, a detector and a data acquisition system as shown in Fig. 1.2. The mobile phase is supplied from one or several reservoirs and flows through the column, usually at a constant rate, and then through the detector[17].

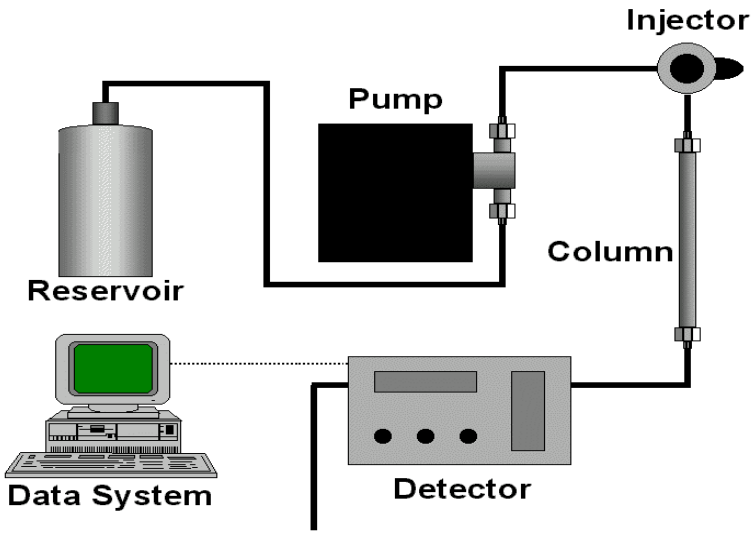


Fig. 1.2 Schematic Flow diagram of HPLC

HPLC can be used for both qualitative and quantitative analysis. In qualitative analysis, the retention time of the compounds are made use of. In quantitative analysis on the other hand, the area under the peak, which is proportional to the concentration of the compound is used.

Pumps

In HPLC, there is the need to deliver a constant flow of the mobile phase. Pumping systems are thus required to deliver the mobile phase at a constant flow rate. Pressure fluctuations are to be minimized as they affect the separation efficiency. The pump must be able to provide pressure of up to 6000 psi, pulse free output, flow rate ranging from 0.1-10 mL/min, flow control and flow reproducibility. Microprocessor controlled systems are capable of accurately delivering a mobile phase of either constant (isocratic elution) or varying composition (gradient elution), according to a defined program. The delivered flow

rate must be independent of the backpressure, even if this changes during a separation, which is usually the case with gradient elution. Moreover, the flow should be pulse less, especially when a refractive index, conductivity or electrochemical detector is used[18].

Injection Systems

Injection ports are of two basic types

- Those in which the sample is injected directly into the column and
- Those in which the sample is deposited before the column inlet and then swept by a valve action into the column by the mobile phase.

On-column injection systems are not as reproducible as the valve injectors and generally are used in older or simple HPLC apparatus. Injectors are further grouped into manual and auto injectors (auto samplers). The manual injectors require the analyst to inject the sample into the valve by a syringe whereas in the auto injectors the injection of the sample is done automatically. Auto samplers eliminate variations due to the analyst, improving reproducibility[19]. Auto samplers can be controlled by computers and are of value where large number of samples are to be analyzed or unattended operation is required. Samples and standards loaded into racks or turntables can be run in a predetermined sequence and under different operating conditions. Such devices can also be used for single samples to improve injection precision.

Columns and Stationary Phases

The column is where the separation process occurs. It is the central component of HPLC. There are many types of stationary phases in HPLC depending on the particular separation technique being employed. Heavy wall glass, stainless steel and plastic are among materials that can withstand high pressures and are used to construct HPLC columns[20]. Columns must not chemically interfere with the mobile phase. Usually a short guard column is placed before the column and this serves to prolong the life of the column by removing particulate matter and contaminants in the solvent.

Detectors

The detector for an HPLC is the component that emits a response due to the eluting samples and subsequently signals a peak on the chromatogram. The detection of the separated components from the column is based upon the bulk property of elute or the solute property of the individual components. There are six main types of detectors used for HPLC: refractive index (RI), ultraviolet (UV), fluorescence (FL), electrochemical (EC), conductivity (CD), and mass spectrometric (MS). Infrared and nuclear magnetic resonance detectors have been used, but they suffer from solvent limitations.

PDA Detector

In Photo Diode Array (PDA) detector, the single detecting element of UV detector has been replaced by an array of solid state detecting elements (photo-diodes). These detectors typically have large numbers of diodes in array (256, 512, and 1024). In PDA, polychromatic radiation, after passing through the sample, is dispersed by a fixed grating and then falls on to an array of photodiodes. Each diode measures a narrow band of wavelengths in the spectrum, thus the PDA has parallel data acquisition, all points in the spectrum being measured simultaneously. The spectrum of each peak in the chromatogram can be stored and subsequently compared with standard spectra, which facilitates the identification of peaks.

This system is superior to other detection systems as:

- There are no moving parts to wear out, wavelength-resetting errors are reduced and the instrument is likely to require less maintenance than does a conventional spectrophotometer
- The ability to make multi wavelength measurements
- Speed of data acquisition means that various signal-averaging techniques can be used to reduce noise
- Improve sensitivity

Peak purity by PDA detector

When two compounds elute very close to each other, they appear as one peak. This can be recognized by a difference of the spectra recorded on the two flags of the peak, provided the two compounds have different absorption spectra. When PDA is combined with a separating column, three dimensions become available wavelength, intensity and chromatographic retention. The spectra obtained must be sufficiently different as specified by their correlation coefficients. Even with a value under 0.9, superimposed peaks can be calculated and evaluated in a three dimensional space without substantial loss in terms of accuracy and precision to know the purity of a particular peak.

PDA Detector was more advantageous compared to other detectors and can easily determine all range of wavelengths and analyze purity of compound. At the column scouting phase, the use of a photo-diode array (PDA) detector will increase the likelihood of detecting degradants with different UV spectrum to that of the API. Thus the purpose present method was used the PDA Detector.

The following diagrams in Fig. 1.3 show the UV and PDA detectors.

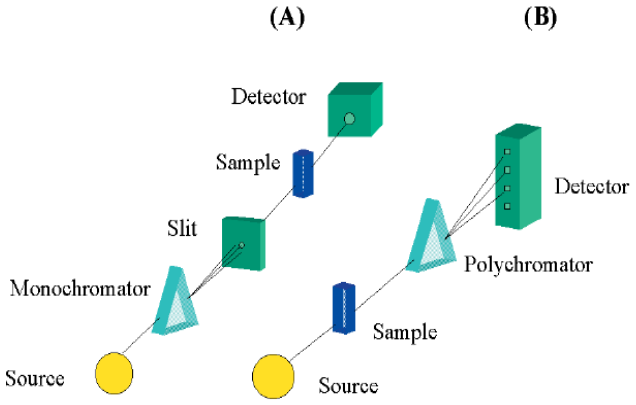


Fig. 1.3 (A) UV Detector; (B) PDA Detector

Reverse-Phase (RP) Chromatography

Reverse phase chromatography is the most widely used form of HPLC. In reverse phase partition chromatography, the stationary phases are non-polar and thus polar mobile phases are required.

Stationary Phase

The stationary phase is silica, chemically bonded through a siloxane linkage to low polar functional group. The surface of the support, e.g. the silanol groups of silica, is reacted with various silane reagents to produce covalently bound silyl derivatives covering a varying number of active sites on the surface of the support[21]. The nature of the bonded phase is an important parameter for determining the separation properties of the chromatographic system. Common reverse phase materials include octadecylsilane (ODS or C_{18}) and octylsilane (C_8).

Mobile Phase

The mobile phase generally comprises of water and a less polar organic solvent modifier such as methanol or acetonitrile. The solutes in reverse phase chromatography are eluted in the order of their decreasing polarities. Separations in these systems are considered to be due to different degrees of hydrophobicity of the solute, the less polar solute partitioning to a greater extent into the non-polar stationary phase and consequently being retained on the column longer than the more polar solute. The rate of elution of the components is controlled by the polarity of the organic modifier and its proportion in the mobile phase. The rate of elution is increased by reducing polarity e.g. by increasing the proportion of the organic solvent or by using acetonitrile instead of methanol.

Commonly used aqueous phases

The Table 1.1 given below shows the commonly used aqueous mobile phases in the HPLC analysis:

Table 1.1 commonly used aqueous phases

Common Buffers		pK_a	Useful pH Range
Phosphate	pK_1	2.1	1.1-3.1
	pK_2	7.2	6.2-8.2
	pK_3	12.3	11.3-13.3
Citrate	pK_1	3.1	2.1-4.1
	pK_2	4.7	3.7-5.7
	pK_3	5.4	4.4-6.4
Formate		3.8	2.8-4.8
Acetate		4.8	3.8-5.8
Tris		8.3	7.3-9.3
Ammonia		9.2	8.2-10.2
Borate		9.2	8.2-10.2
Diethylamine		10.5	9.5-11.5

Solvent Parameters Relating to HPLC

The following are some of the solvent parameters that need to be considered when choosing a suitable mobile phase for HPLC analysis.

UV Transparency and UV cut-off

Most solvents are more transparent to UV down to a certain wavelength and below that they totally absorb UV. To be useful with UV detection, the solvent has to have a lower UV cut off than the absorption of any of the sample components. In general, reverse phase eluents have much lower UV cut-off points than normal phase eluents.

Solvent Miscibility

Some solvents such as alkanes (e.g. hexane, pentane etc) are very non-polar and will not mix with others (such as water), which are highly polar. Since, solvents are mixed in HPLC to fine tune the polarity, thereby controlling their eluent strength, it is essential that solvents chosen are totally miscible.

Viscosity

HPLC operates in dynamic equilibrium. Almost 90% of the surface area of a 5micron packing material is inside the pores. So, the lower the viscosity of a solvent, the lower the backpressure and the better the mass transfer in and out of the pores. This in turn gives better separation efficiency, i.e. sharper peaks.

Purity

For HPLC, solvents used require a higher level of purity. For this purpose, HPLC grade solvents are required. If these are not available, Analytical (AR) grade solvents can be distilled at least once and filtered. The importance of solvent purity is that when analyzing small quantities of sample (20µL), impurities in the 20-30mL of solvent used during a run can be quite significant[22].

Eluent Strength

For Reverse Phase HPLC, water is the weakest eluent[23]. Its eluent strength can be modified by adding a non-polar but miscible solvent such as methanol. Lesser the polarity of the solvent, greater is the eluent strength. For normal phase HPLC, hexane is the weakest eluent and a more polar solvent is added to modify eluent strength. These include chloroform, dichloromethane, ethyl acetate, acetone, ether etc.

If a change in eluent composition is made for a selectivity reasons eg. from methanol to acetonitrile in RP-HPLC, the ratios must be changed to maintain the same eluent strength.

Toxicity

Some solvents are more hazardous than others. Toxicity, flammability, carcinogenicity amongst others is therefore very essential in selecting solvents for HPLC. Some have a very unpleasant odour. Some have a low flash point. It is important to be aware of the hazards.

Cost: Some solvents are very much more expensive than others. For example, for an analysis where methanol or acetonitrile could be used, methanol would be preferred when cost is taken into consideration.

HPLC METHOD DEVELOPMENT:

A good method development strategy should require only as many experimental runs as are necessary to achieve the desired final result. Finally method development should be as simple as possible, and it should allow the use of sophisticated tools such as computer modelling. During initial method development, a set of initial conditions (detector, column, mobile phase) is selected to obtain the first "scouting" chromatograms of the sample. In most cases, these are based on reversed-phase separations on a C_{18} column with UV detection[24].

The important factors, which are to be taken into account to obtain reliable quantitative analysis, are

- Careful sampling and sample preparation.
- Appropriate choice of the column.
- Choice of the operating conditions to obtain the adequate resolution of the mixture.
- Reliable performance of the recording and data handling systems.
- Suitable integration/peak height measurement technique.
- The mode of calculation best suited for the purpose.
- Validation of the developed method.

Before beginning method development, it is needed to review what is known about the sample in order to define the method goals. The following steps are involved in method development.

Sample information

This information is useful for the selection of appropriate sample preparation procedures as well as the initial detection and chromatographic modes. If critical data are not available (e.g., pKa, solubility), separate studies should be initiated as soon as possible. The chemical structure of the analyte furnishes data on molecular weight and the nature of the functional groups. Particular attention should be directed to acidic, basic, aromatic, or reactive functional groups from which estimates of pKa, solubility, chromophoric, or stability data can be inferred. If sufficient purified reference material is available, solubility studies of the analyte in common solvents such as water, alcohol, ether, and hexane should be conducted. Toxicity data and Material Safety Data Sheets (MSDS), COA including spectral data (MS, NMR, IR, and UV) should be ascertained. The chemical composition of the sample can provide valuable clues for the best choice of initial conditions for an HPLC separation.

Chromatographic mode

Reversed-phase chromatography is the most common mode for small organic molecules. Note that ionisable compounds (acids and bases) are often separated by RPC with buffered mobile phases (to keep the analyte in a non-ionized state) or with ion-pairing reagents. In reverse phase mode, the mobile phase is comparatively more polar than the stationary phase. For the separation of polar or moderately polar compounds, the most preferred mode is reverse phase. The nature of the analyte is the primary factor in the selection of the mode of separation. A second factor is the nature of the matrix. Below table gives details of choice of LC mode for different molecules:

Table 1.2 Choice of LC mode

Mode	Solvent type used	Compound type
Reversed Phase	H_2O/Buffer, ACN, MeOH	Neutral or non-ionised compounds which can be dissolved in water/organic mixtures
Ion-Pair RP	Same as above with addition of ion-pair reagent	Ionic or ionisable compounds
Normal Phase	Organic solvents	Mixture of isomers and compounds not soluble in organic/water mixtures
Ion exchange	H_2O/Buffer	Inorganic ions, proteins, nucleic acids, organic acids.
SEC	H_2O, THF, $CHCl_3$, DMF	High molecular weight compounds

Sample preparation

Most samples for HPLC analysis require weighing and /or volumetric dilution before injection. Best results are often obtained when the composition of the sample solvent is close to that of the mobile phase since this minimizes baseline upset and other problems[25]. Some samples require a partial separation (pre-treatment) prior to HPLC, because of need to remove interferences, concentrate sample analytes or eliminate "column killers". Whereas, special samples are better separated under customized conditions given in below table:

Table 1.3 Requirements of Sample Selection

Sample	Requirements
Inorganic ions	Detection is primary problem; use ion chromatography.
Isomers	Some isomers can be separated by reversed-phase HPLC and are then classified as regular samples; better separations of isomers are obtained using either (1) normal-phase HPLC or (2) reversed-phase separations with cyclodextrin-silica columns.
Enantiomers	These compounds require "chiral" conditions for their separation.
Biological	Several factors make samples of this kind "special": molecular conformation, polar functionality, and a wide range of hydrophobicity.
Macromolecules	"Big" molecules require column packing's with large pores (>>10-nm diameters); in addition, biological molecules require special conditions as noted above.

Selection of Column

Selection of the column is the first and the most important step in method development. The appropriate choice of separation column indicates three different approaches[26].

- Selection of separation.
- The particle size and nature of the column packing.
- The physical parameters of the column i.e. the length and the diameter.

Selection of column temperature

Always carry out chromatographic separations at ambient temperature. The increase in column temperature generally will result in reduction of asymmetry and peak retention. The column temperature between 30°C-80°C shall be adopted if necessary. If a column temperature above 80°C is required, packing material which

can with stand to that temperature was preferable. The below table gives an idea on how to select a column:

Table1.4 Selection of Column[27]

Sample		LC mode	Column choice
		Reverse Phase-ion pair (allows neutral and charged compounds to be simultaneously analyzed)	C_{18}, C_8, C_6, C_4, C_2, TMS, CN, amino (not for carbonyl compounds), phenyl, Hamilton PRP-1 (pH 1-13)
Basic or Acidic			
		Ion suppression	C_{18}, C_8, C_6, C_4, C_2, TMS, CN, amino (not for carbonyl compounds), phenyl, Hamilton PRP-1 (pH 1-13)
Ionisable		Ion Exchange	
		Anionic →	Strong Anion exchange
		Cationic →	Strong Cation exchange
		Normal phase	Increasing polarity of bonded phases diol
			CN
Neutral			NH_2
			Silica
		Reverse phase	Alumina, C-$_{18}$, C-$_8$, phenyl

Selection of Detector

The detector was chosen depending upon some characteristic property[28] of the analyte like UV absorbance, florescence, conductance, oxidation, reduction etc.

The characteristics that are to be fulfilled by a detector to be used in HPLC determination are,

- High sensitivity facilitating trace analysis
- Negligible baseline noise to facilitate lower detection
- Large linear dynamic range
- Low dead volume
- Ease in calibration and standardization
- Inexpensive to purchase and operate

Pharmaceutical ingredients do not absorb all UV light equally, so that selection of detection wavelength is important. An understanding of the UV light absorptive properties of the organic impurities and the active pharmaceutical ingredient is very helpful. Generally LC equipped with PDA detector was the first choice.

For the greatest sensitivity λ_{max} should be used. Ultra violet wavelengths below 200nm should be avoided because detector noise increases in this region. Higher wavelengths give greater selectivity.

METHOD VALIDATION

Analytical method validation is a process of performing several tests designed to verify that an analytical test system is suitable for its intended purpose and is capable of providing useful and valid analytical data. A validation study involves testing multiple attributes of a method to determine that it can provide useful and valid data when used routinely. There are several parameters that are considered in the method validation process as per International Conference of Harmonization (ICH) Guidelines and are as follows:

Method validation

a. Specificity
b. Linearity
c. Precision
    i. System Precision
    ii. Method Precision
d. Accuracy
e. Robustness
f. LOD and LOQ
g. System suitability
h. Stability of the solution
i. Assay

Specificity

Specificity studies were carried for the pure drug and drug product by comparing the 3D plots with blank and placebo. Peak purity tests were also carried out to show that the analyte chromatographic peak is not attributable to more than one component as the impurities are not available by purity index data. The data was shown in Figures 3.1- 3.3.

Linearity

A linear relationship was evaluated across the range of the analytical procedure with a minimum of five concentrations. A series of combination standard dilutions were prepared over a concentration range of 10-50µg/mL for Minocycline hydrochloride from stock solution of the pure drug and injected in triplicate. Linearity is evaluated by a plot of peak areas as a function of analyte concentration,

and the test results were evaluated by appropriate statistical methods where by slope, intercept, and regression (R^2) and correlation coefficient (R) were calculated and the data was given in Table 3.1 and shown in Figures 3.4-3.10.

Precision

Precision is the measure of closeness of the data values to each other for a number of measurements under the same analytical conditions. Repeatability was assessed by using a minimum of six determinations at 100 % of the test concentration (30µg/mL of Minocycline hydrochloride). The standard deviation and the relative standard deviation were reported for precision. Less than 2 % RSD for peak areas and 1% RSD for retention times of the drug indicates the precision of the developed method and the data was presented in Table 3.2, 3.3 and shown in Figures 3.11-3.24.

Accuracy

Accuracy was established across the specified range of the analytical procedure. To ascertain the accuracy of the proposed method recovery studies were performed by the standard addition method by spiking 80%, 100%, 120% of the known quantities of standards within the range of linearity to the synthetic solution of drug product (10µg/mL of Minocycline hydrochloride) and these mixture solutions were analyzed by developed method in triplicate. The % Recovery and the % RSD were calculated for both the drugs at each level of addition and the data was given in Table 3.4 and depicted in Figures 3.25-3.33.

LOD and LOQ

LOD and LOQ were calculated based on calibration curves. They were expressed as LOD = (3.3 ×σ)/m; LOQ= (10.0×σ)/m (Where, σ is the standard deviation of the y-intercepts of the three regression lines and m is mean of the slopes of the three calibration curves).

Robustness

To determine the robustness of the method developed, the experimental conditions were deliberately altered and the chromatographic parameters viz., tailing factor, no. of theoretical plates were recorded. The flow rate of the mobile phase was 1.2mL/min. To study the effect of flow rate, on the proposed method, the flow rate was changed to 10% and the effect of wavelength was studied by changing wavelength by ±1nm and the data was given in Table 3.5 and shown in Figures 3.34-3.39.

System Suitability

Increment volumes (10-50µL) of standard concentration were injected to assess the system suitability. The data was given in Table 3.6 and shown in Figures 3.40-3.45.

Stability of the solution

The stability of the stock and standard solutions were determined by analyzing the samples under refrigeration (8±1°C) at different time intervals up to 24 hours. The data was given in Table 3.7 and shown in Figures 3.46-3.47.

Assay

Twenty tablets were weighed and finely powdered, the powder blend equivalent to 10mg of Minocycline hydrochloride was accurately weighed and transferred into a 10 mL volumetric flask and dissolved in methanol and vortexed for 5 min and volume was adjusted up to the mark with methanol to get 1mg/mL of Minocycline hydrochloride. The above solution was centrifuged and then filtered using Nylon disposable syringe filter (0.45 µm) and the 10µL of filtrate was diluted with ammonium acetate to 1mL to get a solution containing 10µg/mL of Minocycline hydrochloride. The solution was injected in triplicate and analyzed. The amount present in each tablet was quantified by comparing the area of standard Minocycline hydrochloride with that of the sample. The data was given in Table 3.8 and shown in Figure 3.48-3.50.

Calculation:

Amount of Minocycline hydrochloride per tablet was calculated by using the following formula:

$$\text{Minocycline hydrochloride (mg/ tablet)} = \frac{A_T}{A_{ST}} \times \frac{D_S}{D_T} \times \frac{P}{100}$$

Where,

A_T = Average area counts of injections for Minocycline hydrochloride peak in the chromatogram of sample solution.

A_{ST} = Average area count of three replicate injections for Minocycline hydrochloride Peak in the chromatogram of standard solution

D_S = Dilution factor of standard solution

D_T = Dilution factor of sample solution

P = Percentage purity of working standard used

$$\% \text{ Labelled Amount} = \frac{\text{Content of each Minocycline hydrochloride (mg/tablet)}}{\text{Label claim, in mg}} \times 100$$

2. LITERATURE REVIEW

The analytical methods reported so far for the estimation of Minocycline hydrochloride individually include HPLC[3-5], UPLC[6], UV Spectrophotometry[7,8], LC-MS/MS[9,10] etc. Following is the summary of the HPLC, UPLC methods for the analysis of Minocycline hydrochloride in bulk/formulation and in biological fluids.

Report on Literature Survey:

Literature survey reveals that only few HPLC methods were reported for the quantitative determination of Minocycline hydrochloride but there were no validated RP-HPLC/PDA methods reported for the estimation of Minocycline hydrochloride in bulk, pharmaceutical dosage forms.

Hence, the present investigation was aimed at developing a validated RP-HPLC-PDA method for the analysis of Minocycline hydrochloride in bulk, pharmaceutical dosage forms which is LC-MS compatible, accurate, precise and economical.

1. ***Alka Agarwal et., al[27]***, was developed in simple, fast, precise and accurate high performance liquid chromatographic method has been developed for the quantitative estimation of minocycline hydrochloride from powder for oral suspension formulation. The method was developed using acetonitrile : Phosphate buffer in the proportion of 25 : 75 v/v at flow rate of 0.5 mL/min as mobile phase. The separation was carried out on Inertsil ODS 3V C18 (250 mm x 4.6 mm, 5 µm) column and the eluents were detected at 280 nm. The linearity was observed in the concentration range of 100 to 900 µg/mL of minocycline hydrochloride. The LOD and LOQ of the method were 0.816 and 2.474 µg/mL, respectively.

2. ***S. Talegaonkar et., al[28]*** , HPTLC method has been established for determination of minocycline in human plasma. Chromatography was performed on aluminium plates coated with silica gel 60F254; the mobile phase was methanol–acetonitrile–isopropanol–water 5:4:0.5:0.5 (v/v). Densitometric analysis was performed at 345 nm. The method is rapid (single-step extraction

with methanol), sensitive (limit of quantification 15.4 ng per zone), precise (CV ≤ 4.61 %), accurate (drug recovery 95.08–100.6%), and linear over the range 100–1200 ng per zone. Recovery of minocycline from plasma samples was 95.8 ± 4.5%. The halflife of minocycline in plasma was 9.9 h at 4°C and 6.3 h at 20°C. Minocycline is stable in human plasma for at least two months at −20°C and can tolerate two freeze–thaw cycles with losses.

3. ***Wenna Shi, Zuozhong chen, et., al[41]***, Combination therapy can be used for the treatment of fungal infections, especially for those caused by antifungal-resistant fungi. In the present study, *in vitro* interactions and mechanisms between fluconazole and minocycline against *Candida albicans* were evaluated. The nature of the interactions determined by spectrophotometric method in a checkerboard assay was interpreted using nonparametric models of fractional inhibitory concentration index (FICI) and percentages of growth difference (Δ*E*). In the mechanism study, we evaluated the potential activity of minocycline on fluconazole penetrating the *C. albicans* biofilm. Furthermore, the effect of fluconazole and minocycline alone and in combination on the cellular calcium balance, as well as on the uptake and efflux of fluconazole were evaluated. It was found that fluconazole can work synergistically with minocycline against fluconazole-resistant *C. albicans*; the minimum inhibitory concentration of fluconazole decreased from 512 to 2 μg mL^{-1} when fluconazole and minocycline were given in combination, with an FICI of 0.035 and 0.064 and high-percentage synergistic interactions of 1250% and 988% for the two resistant strains. The mechanism of action was suggested to be the enhancement of minocycline on fluconazole penetrating biofilm, and inducing the intracellular calcium release, instead of impacting on the uptake and efflux of fluconazole.

4. ***Victoria F. Samanidou et., al[30]***, A high-performance liquid chromatographic method with diode-array detection, at 351 nm, was developed and validated for the determination of five tetracyclines (TCs): minocycline, tetracycline, oxytetracycline, chlortetracycline, and doxycycline in bovine muscle. Samples were macerated with a buffer solution, centrifuged, and purified using Abselut Nexus SPE cartridges. The separation of the examined TCs was achieved on an

Inertsil ODS-3 5 μm, 250×4 mm analytical column, at ambient temperature. A multistep gradient elution was followed using 0.05 M oxalic acid and CH_3CN, at a flow rate of 1.65 mL/min. The procedure was validated according to the European Union regulation 2002/657/EC determining selectivity, stability, decision limit, detection capability, accuracy, and precision.

5. ***Gaurav K Jain et., al[34]***, high-performance thin-layer chromatographic (HPTLC) me-thod has been established for determination of minocycline in human plas-ma. Chromatography was performed on aluminium plates coated with silica gel 60F 254 ; the mobile phase was methanol–acetonitrile–isopropanol–wa-ter 5:4:0.5:0.5 (v/v). Densitometric analysis was performed at 345 nm. The method is rapid (single-step extraction with methanol), sensitive (limit of quantification 15.4 ng per zone), precise (CV ≤ 4.61 %), accurate (drug recovery 95.08–100.6%), and linear over the range 100–1200 ng per zone. Recovery of minocycline from plasma samples was 95.8 ± 4.5%. The half-life of minocycline in plasma was 9.9 h at 4°C and 6.3 h at 20°C. Minocy-cline is stable in human plasma for at least two months at −20°C and can tolerate two freeze–thaw cycles with losses <10%.

6. ***M.Thakkar et., al[33]***, precise and accurate high performance liquid chromatographic method has been developed for the quantitative estimation of minocycline hydrochloride from powder for oral suspension formulation. The method was developed using acetonitrile: Phosphate buffer in the proportion of 25: 75 v/v at flow rate of 0.5 mL/min as mobile phase. The separation was carried out on Inertsil ODS 3V C18 (250 mm x 4.6 mm, 5 μm) column and the eluents were detected at 280 nm. The linearity was observed in the concentration range of 100 to 900 μg/mL of minocycline hydrochloride. The LOD and LOQ of the method were 0.816 and 2.474 μg/mL, respectively.

7. ***Adapa V.S.S Prasad et., al[35],*** *was developed in* Sensitive spectrophotometric methods (M_1–M_4) by the application of oxidative coupling and diazocoupling reactions for the assay of minocycline (MC) in pure form and pharmaceutical formulations have been described. Methods M_1 and M_2involve the oxidative coupling reactions of MC with 3-methyl-2-benzothiozolinone hydrazone

(MBTH) (method M_1, λ_{max} 440 nm) or 4-aminophenazone (4-AP) (method M_2, λ_{max} 520 nm) in the presence of periodate. Methods M_3 and M_4 are based on the formation of diazocoupling products of MC with diazotised *p*-nitroaniline (DPNA) (method M_3, λ_{max} 420 nm) or diazotised sulfanilic acid (DSAC) (method M_4, λ_{max} 420 nm). Regression analysis of Beer's law plot showed good correlation in the concentration range of 8–48, 20–120, 4–20 and 8–40 μg ml^{-1} for methods A, B, C and D, respectively.

8. ***E. Rosier, et., al[29]*** , was developed in human and animal remains by means of analysis of volatile compounds released during decomposition is impossible since no volatile marker(s) specific for human decomposition has been established today. Hence, the identification of such a marker for human decomposition would represent great progression for the discovery of buried cadavers by analytical techniques. Cadaver dogs can be trained more efficiently, the understanding of forensic entomology can be enhanced, and the development of a portable detection device may be within reach. This study describes the development and validation of a new analytical method that can be applied in the search of such (a) specific marker(s). Sampling of the volatile compounds released by decomposing animal and human remains was performed both in a laboratory environment and outdoors by adsorption on sorbent tubes. Different coatings and several sampling parameters were investigated.

9. ***Slavica M.Sunarić[ab]Marko S.Denić et., al[31]***, was developed in the present paper reports the development and validation of an analytical method for doxycycline quantification in human seminal fluid by HPLC with UV detection. The separation of doxycycline was achieved at 40 °C on a reversed-phase C18 column using isocratic elution. The mobile phase consisted of acetonitrile (A) and water buffered at pH 2.5 with a concentrated orthophosphoric acid (B) in the volume ratio of 20:80 (v/v), respectively. The detection was performed at 350 nm. As an internal standard (IS), tetracycline was used. The proposed method involves the extraction of doxycycline from seminal fluid based on acidic precipitation of the proteins using perchloric acid. The method showed good intra- and inter-day precisions (RSD < 7.0%), good accuracy (recovery for

doxycycline > 80%), and high correlation coefficient (r = 0.998) for standards subjected to the entire procedure. The detection and quantification limits were 0.087 μg/ml and 0.264 μg/ml. The developed method was used to analyze doxycycline in the seminal fluids obtained from male subjects who were treated with doxycycline-hyclate. The mean doxycycline concentrations of 0.89 ± 0.07 μg/ml and 0.45 ± 0.26 μg/ml were detected in seminal fluid after 6 h and 12 h, respectively.

10. ***S.Sharma et., al***[32], New, simple, precise, and accurate HPTLC method for simultaneous quantitation of Metronidazole (MET) and and Tetracycline hydrochloride (TET) as the bulk drug and in capsule dosage forms have been developed. Chromatographic separation of the drugs was performed on aluminium plates precoated with silica gel 60 F254 as the stationary phase and the solvent system consisted of benzene: ethyl acetate: toluene: methanol: glacial acetic acid (9.5:2.0:5.0:1.5:0.5 v/v/v/v/v),Densitometric evaluation of the separated zones was performed at 254 nm. The two drugs were satisfactorily resolved with R f values of 0.43 ± 0.02 and 0.74± 0.02 for MET and TET, and 0.29 Ofloxacin (OF) respectively.

11. ***Naidong Weng et., al***[36] was developed in liquid chromatographic methods laid out for the analysis of minocycline hydrochloride in the British Pharmacopoeia 1988, the United States Pharmacopeia XXII and the Pharmacope Franaise 10 were examined. The major difference between these methods lies in the adjustment of the pH of the mobile phase. The analyst has to obtain the right pH of the mobile phase for his available column. This was found to be a less than easy task for the occasional analyst. Other disadvantages observed were poor solubility of the components of the mobile phase, reduced sensitivity due to UV absorbance by the mobile phase, rather high back pressure, the elution order for 9-minocycline depending on the brand of stationary phase, and above all, poor stability of the stationary phase.

12. ***Victoria F Samanidou et., al***[37] confirmatory high-pressure liquid chromatographic method for the determination of nine tetracyclines in Sparus aurata (gilthead seabream) muscle tissue is developed and presented herein.

Tetracycline, 4-epi-tetracycline, oxytetracycline, 4-epi-oxytetracycline, chlortetracycline, 4-epi-chlortetracycline, doxycycline, methacycline and demeclocycline were separated on a Kromasil, C18 (250 mm × 4 mm, 5 μm) analytical column by gradient elution with a mobile phase consisting of 0.001 M ethylenediaminetetraacetic acid/sodium salt and acetonitrile at 25°C. Diode array detection with monitoring at 280 nm (for the determination of chlortetracycline, 4-epi-chlortetracycline, methacycline and demeclocycline) and 355 nm (for tetracycline, 4-epi-tetracycline, oxytetracycline, 4-epi-oxytetracycline and demeclocycline) was applied for peak identification and quantification of analytes.

13. ***Mukesh Kumar Raikwar et., al[38]*** To develop and validate method for tetracycline antibiotics and their epimers in marine products as per EU commission (2007/657/EC). Now a day it becomes necessary to detect tetracycline as well as their epimers at EUMRL level with high confidence so that effort was made to separate and detect tetracyclines (tetracycline, oxytetracycline, doxytetracyclinea and chlortetracycline) and their epimers (epitetracycline, epichlortetracycline and epioxytetracycline). Here isomers have the same molecular weight and somewhat chemical nature too so that long run time was used to get separate peaks. For sample extraction Mcvalline buffer was used and then extract was purified by passing through a C18 solid phase extraction (SPE) column. The prepared samples were analyzed on LC/MSMS. Tetracycline antibiotic residues in marine products were analyzed. The recovery rates of seven tetracyclines in sample preparation ranged from 77.03 % to 89.95 % in fortified blanks; the coefficient variation were between 0.35 % and 2.89 %. The Decision limits (CCα) were 102.93 to 104.23 ng/g and Detection Capability (CCβ) was 105.78 to 108.26 ng/g.

14. ***Ewelina Patyra et., al[39]***, A chromatographic procedure for the determination of oxytetracycline (OTC), tetracycline (TC), chlorotetracycline (CTC), and doxycycline (DC) in medicated feedingstuffs was developed. Samples were extracted with 0.01 M citric buffer/acetonitrile (pH 3.0) and further purified with 0.45 μm syringe filters. The purified extract was separated on Thermo column C_{18}, 150 × 4 mm, 5 μm and detection was carried out at 360 nm for

OTC, and TC, 370 nm for CTC, and 350 nm for DC. TCs were eluted with a mobile phase of 0.03 M SDS/7 % 1-butanol/0.02 M oxalic acid/NaOH at pH 2.5. This method provided average recoveries of 80.4 % to 100.2 %, with CVs of 0.5 % to 6.6 % in the range of 50 to 1500 mg/kg OTC, TC, CTC, and DC in feeds. The linearity for the four TCs was determined by high-performance liquid chromatography-diode array detector (HPLC-DAD) in the range 10–300 μg/mL (50–1500 mg/kg), with a linear correlation coefficient (R) > 0.99. The LOD and LOQ for TCs in pig and poultry feeds ranged from 4.0 to 10.7 and 4.7 to 12.6 mg/kg, respectively.

15. ***Emad M. Hussien et., al***[40], Novel reversed-phase HPLC method was developed and validated for the assay of tetracycline hydrochloride and the limit of 4-epianhydrotetracycline hydrochloride impurity in tetracycline hydrochloride commercial bulk and pharmaceutical products. The method employed L1 (3 μm, 150 × 4.6 mm) columns, a mobile phase of 0.1% phosphoric acid and acetonitrile at a flow rate of 1.0 mL/min, and detection at 280 nm. The separation was performed in HPLC gradient mode. Forced degradation studies showed that tetracycline eluted as a spectrally pure peak and was well resolved from its degradation products. The fast degradation of tetracycline hydrochloride and 4-epianhydrotetracycline hydrochloride in solution was retarded by controlling the autosampler temperature at 4 °C and using 0.1% H_3PO_4 as diluent. The robustness of the method was tested starting with the maximum variations allowed in the *US Pharmacopeia* (*USP*) general chapter *Chromatography* <621>.

3. OBJECTIVES AND PLAN OF WORK

In modern days usage of large number of drugs is increased to save the life of human beings, to control the spreading of large number of diseases around the world.

A drug is having many physical and chemical properties that show variation in properties at various stages of bulk drug manufacturing, synthesis stage and formulation stage. These properties are estimated by qualitative and quantitative approach by using various number of new techniques.

Modern pharmaceutical industries generally require precise analytical instruments at very low concentrations with various types of instruments like HPLC, LC-MS, UP-LC, GC, NMR and IR[1]. Using these instrumental techniques high resolution can be achieved. There is continuous change in techniques in the method development for various numbers of drugs for the quantification purpose are used presently. There are often a huge number of alternative methods for solving any analytical method development, but by understanding the advantages and limitations of the various tools we can choose the most appropriate instrumental method to achieve the limitations in sensitivity, precision, and accuracy.

Various number of combination drug formulations are released in to the market to reduce the side effects and to minimize the drug toxicity. In these formulations resolution has to be done to determine the content of drug present in it. The advanced instruments like LC-MS and UP-LC are highly economic to develop the new methods used in industry.

Many analytical techniques are used in the pharmaceutical industry to characterize a diversity of substances including active ingredients, impurities, counter-ions, excipients, extractable and process contaminants. HPLC is widely used in the pharmaceutical industry for quantitative analysis of a broad range of substances. While UV remains a primary detection technique, many analytes of interest (e.g., active pharmaceutical ingredient (API), impurities, counter-ions, excipients, extractables, and contaminants) lack a sufficient UV chromophore. Furthermore, the response obtained with UV and many other detectors is highly

dependent on analyte properties. This posses significant challenges since individual standards are not always available, or are impractical, to use for calibration. Hence number of companies are mainly focusing on HPLC methods which are simple, precise, economical and efficient for the estimation purpose.

The Validation of Analytical Procedures (Q_2R_1) issued by International Conference on Harmonization (ICH) requires that analytical test procedures should be fully validated and the analytical procedures should be investigated in order to ensure the quality of the drug substance or drug product[2].

The main aim of the present investigation is to develop a validated RP-HPLC-PDA method for the estimation of Minocycline Hcl in bulk, pharmaceutical dosage form, rapid, sensitive and cost effective method.

4. DRUG PROFILE

MINOCYCLINE HYDROCHLORIDE

Minocycline hydrochloride is a Broad spectrum tetracycline antibiotic. It is used to treat acne vulgaris. Chemically it is (4S,4aS,5aR,12aR)-4,7-bis(dimethylamino)-1,10,11,12a-tetrahydroxy-3,12-dioxo-3,4,4a,5,5a,6,12,12a-octahydrotetracene-2- carboximidicacid hydrochloride.

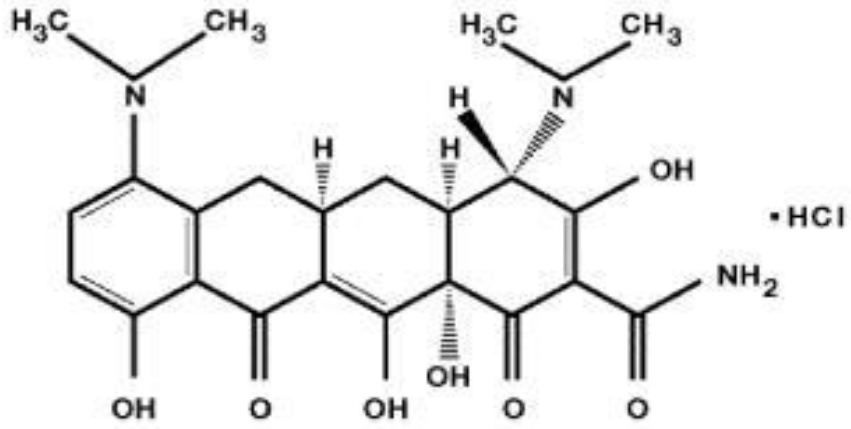


Fig. 1.1 Chemical structure of Minocycline hydrochloride

Physicochemical properties of minocycline hydrochloride

Description : Broad spectrum tetracycline antibiotic

Mol. Mass : 493.937g/mol

Mol. Formula : $C_{23}H_{28}ClN_3O_7$

Melting point : 205-210^{0}C

P^{ka} : Strongest Acidic (-1.1), Strongest Basic (10.87)

Solubility : Sparingly soluble in water, slightly soluble in alcohol. It dissolves in solutions of alkali hydroxides and carbonates

Storage : In air tight container, protected from light.

Brand Names : Minocin, Solodyn, Dynacin

Pharmacological properties of minocycline hydrochloride

Mechanism of action[13]

Minocycline passes directly through the lipid bilayer or passively diffuses through porin channels in the bacterial membrane. Tetracyclines like minocycline bind to the 30S ribosomal subunit, preventing the binding of tRNA to the mRNA-ribosome complex and interfering with protein synthesis.

Pharmacokinetics[14]

Absorption	: Rapidly absorbed from the gastrointestinal tract and absorption is not significantly impaired by ingestion of food or milk. Oral bioavailability is 100%.
Protein binding	: 55% to 76
Metabolism	: Hepatic
Excretion	: Mostly fecal rest renal
Half life	: 11-22hrs
Route administration	: Oral, i.v
Dose	: 50 to 100mg

Indications:

For the treatment of infections caused by susceptible strains of microorganisms, such as Rocky Mountain spotted fever, typhus fever and the typhus group, Q fever, rickettsial pox and tick fevers caused by Rickettsiae, upper respiratory tract infections caused by *Streptococcus pneumoniae* and for the treatment of asymptomatic carriers of *Neisseria meningitidis*.

Adverse effects:

The common side effects include nausea, vomiting, diarrhea, dizziness, drowsiness, mouth sores, and cough, discoloration of skin and nails, headache. Other side effects include

- kidney problems - little or no urinating; painful or difficult urination; swelling in your feet or ankles; feeling tired or short of breath;
- liver or pancreas problems - nausea, vomiting, upper stomach pain that may spread to your back, tired feeling, loss of appetite, dark urine, clay-colored stools, jaundice (yellowing of the skin or eyes);
- increased pressure inside the skull - severe headaches, ringing in your ears, dizziness, vision problems, pain behind your eyes;
- signs of inflammation in your body - swollen glands, flu symptoms, easy bruising or bleeding, severe tingling or numbness, muscle weakness, chest pain, new or worsening cough with fever, trouble breathing; or
- severe skin reaction - fever, sore throat, swelling in your face or tongue, burning in your eyes, skin pain, followed by a red or purple skin rash that spreads (especially in the face or upper body) and causes blistering and peeling.

Toxicity

Minocycline has been observed to cause a dark discoloration of the thyroid in experimental animals (rats, minipigs, dogs and monkeys). In the rat, chronic treatment with minocycline has resulted in goiter accompanied by elevated radioactive iodine uptake and evidence of thyroid tumor production. Minocycline has also been found to produce thyroid hyperplasia in rats and dogs. LD_{50}=2380 mg/kg (rat, oral), LD_{50}=3600 mg/kg (mouse, oral).

5. MATERIALS AND METHODS

Reagents and Chemicals

- Minocycline hydrochloride (Dr. Reddy's Laboratories. Hyderabad)
- Water - HPLC grade (E. Merck, Mumbai)
- Ammonium Acetate- HPLC grade (E. Merck, Mumbai)
- Methanol- HPLC grade (E. Merck, Mumbai)
- Acetonitrile - HPLC grade (E. Merck, Mumbai)
- Sodium Lauryl Sulphate (Loba Chemie, Mumbai)
- Divaine tablets 50mg (AH3445) were commercially purchased.

Instrumentation

Quantitative RP-HPLC was performed on a high pressure gradient High Performance Liquid Chromatography (Shimadzu HPLC, class VP series) with two LC-20AD pumps, SIL-20A auto sampler was used with 200µL loop volume, programmable variable wavelength PDA detector SPD-M20A VP, and Agilent Eclipse C_{18} column (150×4.6 mm and 5 µm particle size). The HPLC system was equipped with "LC-Solution" software to acquire and process the data. The instrument is shown in the following Fig. 2.1.

Fig. 2.1 Shimadzu Prominence HPLC with *SIL-20A auto sampler* and *SPD-M20A VP PDA detector*

Preparation of stock and standard solutions

Standard stock solution of Minocycline hydrochloride 1 mg/mL was prepared using HPLC grade methanol 10 mg of Minocycline hydrochloride was dissolved in 10 mL of methanol (1 mg/mL) solution

Appropriate volumes (10-50 µL of Minocycline hydrochloride) of this stock solution were then further diluted with ammonium acetate to 1 mL to get the required concentrations of standard solutions at a concentration range of 10-50 µg/mL.

METHOD DEVELOPMENT

The objective of this experiment was to optimize the RP-HPLC-PDA method for the estimation of Minocycline hydrochloride, based on the literature survey made.

In developing this method, a systematic study of effects of various parameters was under taken by varying one parameter at a time and controlling all other parameters.

A) Selection of Stationary phase

B) Selection of Mobile phase

C) Selection of wavelength

The following trials were carried out in order to optimize the LC conditions for the estimation of Minocycline hydrochloride in bulk and pharmaceutical dosage forms for further validation of the method conditions and *in vitro* dissolution samples analysis

Trial-1

The following are the chromatographic conditions used in the initial trial. The mobile phase optimization was initiated using HPLC water and methanol as organic phase.

Preparation of 0.02%V/v/v Formic acid: 0.02ml of formic acid was accurately weighed, transferred to a clean dry volumetric flask containing 100 mL of HPLC grade water, dissolved and the volume was adjusted to 500 mL with the same solvent.

Chromatographic conditions:

Mobile phase	HPLC Water:Methanol(50:50%v/v)
Flow rate	1.0 mL/min
Column	Inertsil ODS Column (150x4.6 mm, 5µ)
Detector wave length	220 nm
Column temperature	Ambient
Injection volume	10 µL
Run time	15 min
Diluent	Water

Below is the minocycline hydrochloride chromatogram obtained with these conditions:

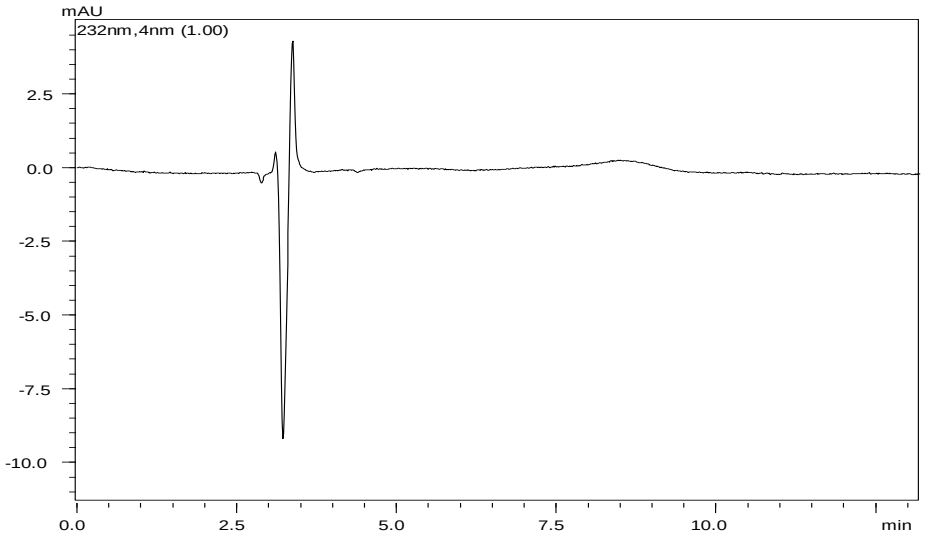


Fig. 2.2 Trial - 1 Chromatogram of standard Minocycline hydrochloride

S. No	Name of Peak	Retention time (min)
1	Minocycline hydrochloride	No peak eluted

Observation:

Under these conditions, no peak eluted

Trial-2

In this trial, except for the change in organic phase to 0.02%V/v/v Formic acid:Acetonitrile in ratio (80:20v/v) and all other conditions were maintained as above.

Chromatographic conditions:

Mobile phase	0.02%V/v/v Formic acid:Acetonitrile (80:20v/v)
Flow rate	1.0 mL/min
Column	Inertsil ODS Column (150x4.6 mm, 5µ)
Detector wave length	220 nm
Column temperature	Ambient
Injection volume	10 µL
Run time	7 minutes
Diluent	Water

Below is the minocycline hydrochloride chromatogram obtained with these conditions:

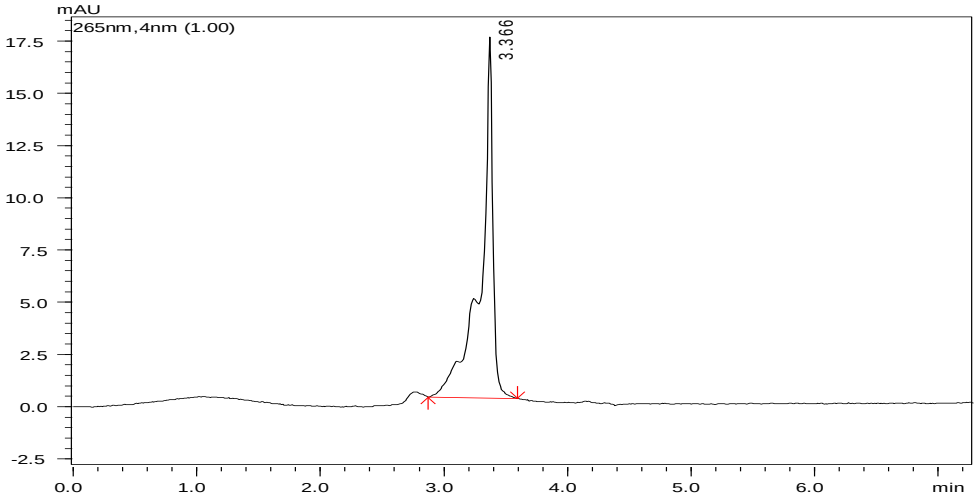


Fig. 2.3 Trial - 2 Chromatogram of standard Minocycline hydrochloride

S.NO	Name of the peak	Retention time (min)
1	Minocycline hydrochloride	3.36

Observation:

Under these minocycline hydrochloride conditions, eluted with retention time of 3.36min but peak fronting was observed.

Trial-3

In this trial, except for the change in the ratio of organic phase (85:15 v/v) all the other conditions were maintained as above.

Chromatographic conditions:

Mobile phase	0.02%V/v/v Formic acid:Acetonitrile (85:15v/v)
Flow rate	1.0 mL/min
Column	Inertsil ODS Column (150x4.6 mm, 5µ)
Detector wave length	220 nm
Column temperature	Ambient
Injection volume	10 µL
Run time	10 min
Diluent	Water

Below is the minocycline hydrochloride chromatogram obtained with these conditions:

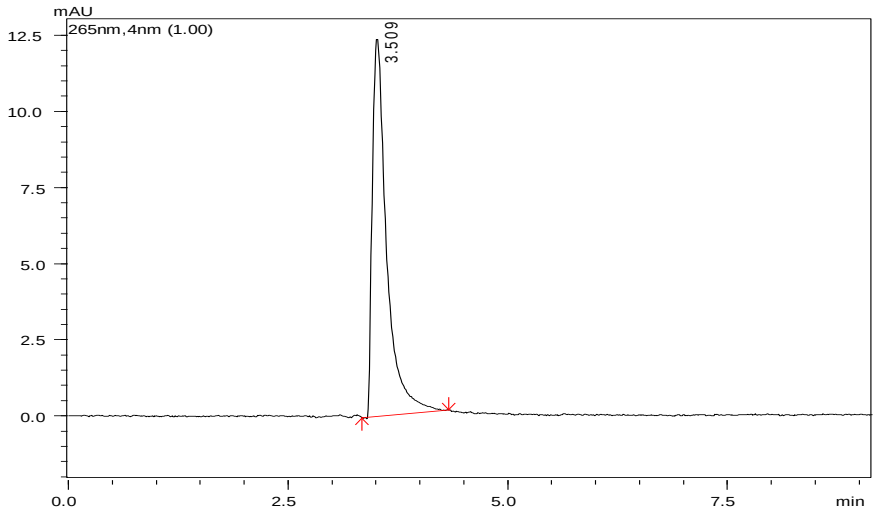


Fig. 2.4 Trial - 3 Chromatogram of standard Minocycline hydrochloride

S.NO	Name of the peak	Retention time (min)
1	Minocycline hydrochloride	3.50

Observation:

Under these conditions, Minocycline hydrochloride eluted at retention time of 3.50min, but the peak symmetry was not good and baseline drift was observed. By observing this it can be concluded that by increasing the ratio of organic phase, retention time was increased but the symmetry could not be improved.

Trial-4

In this trial, except for the change in the ratio of organic phase (90:10 v/v) and all the other conditions were maintained as above.

Chromatographic conditions:

Mobile phase	0.02%V/v/v Formic acid:Acetonitrile (90:10v/v)
Flow rate	1.0 mL/min
Column	Inertsil ODS Column (150x4.6 mm, 5µ)
Detector wave length	220 nm
Column temperature	Ambient
Injection volume	10 µL
Run time	7 min
Diluent	Acetonitrile

Below is the minocycline hydrochloride chromatogram obtained with these conditions:

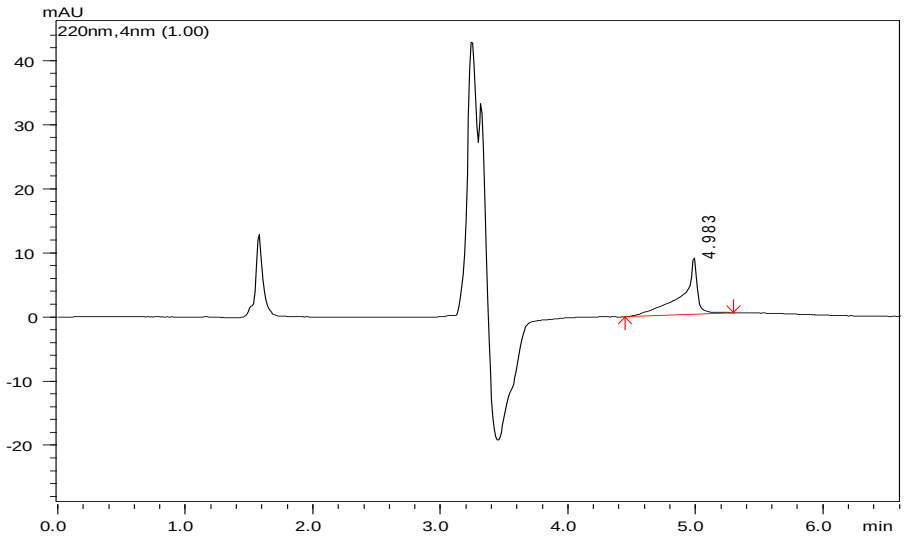


Fig. 2.5 Trial - 4 Chromatogram of standard Minocycline hydrochloride

S.NO	Name of the peak	Retention time (min)
1	Minocycline hydrochloride	4.98

Observation:

Under these conditions, Minocycline hydrochloride eluted at the retention time of 4.98min, with peak fronting and broad peak shape. By observing this it can be concluded that even by changing the buffer strength of mobile phase, fronting could not be prevented.

Trial-5

In this trial, except for the change in organic phase 0.02%V/v/v formic acid: Methanol (70:30 v/v) and all the other conditions were maintained as above.

Chromatographic conditions:

Mobile phase	**0.02%V/v/v Formic acid:Methanol (70:30v/v)**
Flow rate	1.0 mL/min
Column	Inertsil ODS Column (150x4.6 mm, 5µ)
Detector wave length	220 nm
Column temperature	Ambient
Injection volume	10µL
Run time	7 min
Diluent	Water

Below is the minocycline hydrochloride chromatogram obtained with these conditions:

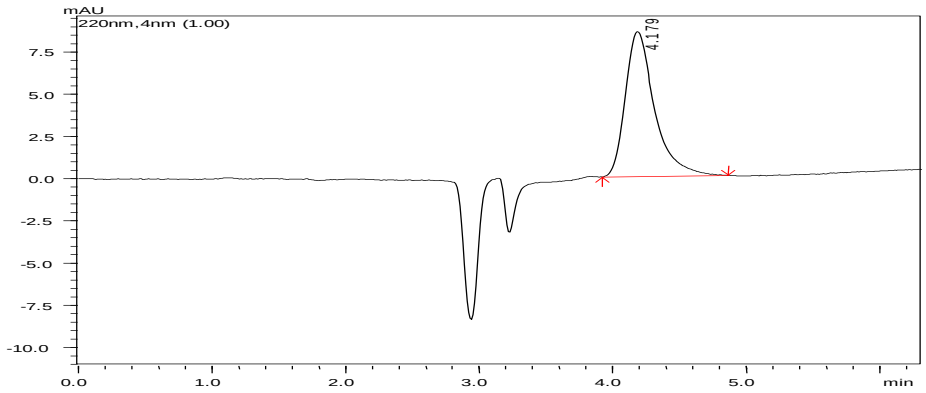


Fig. 2.6 Trial - 5 Chromatogram of standard Minocycline hydrochloride

S.NO	Name of the peak	Retention time (min)
1	Minocycline hydrochloride	4.17

Observation:

Under these conditions, minocycline hydrochloride eluted at 4.17min with tailing factor below 2 and good peak symmetry. Hence the above conditions were established as the optimized method.

Parameters of optimized method:

Below are the parameters obtained with optimized LC conditions:

Table 2.1 Parameters of optimized method

Parameters	Observation
	Minocycline hydrochloride
Retention time	4.17
Peak area	132583
Theoretical plates	2050.591
Tailing factor	1.695

Acceptance criteria:

- The theoretical plate number should be greater than 2000
- The tailing factor should be less than 2

Conclusion:

- ✓ The parameters of minocycline hydrochloride peak obtained with the optimised method were found to be with in the acceptance criteria.
- ✓ The retention time was satisfactory with shorter runtime and makes the proposed method was economical.
- ✓ The peak shape was good and symmetric.

Based on the peak parameters obtained, these LC conditions were further used in method validation.

6. RESULTS AND DISCUSSION

Minocycline hydrochloride is a Broad spectrum tetracycline antibiotic. Chemically it is (4S,4aS,5aR,12aR)-4,7-bis(dimethylamino)-1,10,11,12a-tetrahydroxy-3,12-dioxo3,4,4a,5, 5a,6, 12,12a-octahydrotetracene-2-carboximidic acid hydrochloride and is used to treat Acne Vulgaris.

Analytical methods have been reported in the literature for quantitative determination of Minocycline Hcl by HPLC, UPLC, UV Spectroscopy, and by LC-MS. However, the so far reported HPLC methods for the estimation of Minocycline hydrochloride in bulk and pharmaceutical dosage forms used mobile phase combination of phosphate buffer and acetonitrile. Literature survey reveals that there were no validated RP-HPLC/PDA methods reported for the estimation of Minocycline hydrochloride in bulk, pharmaceutical dosage forms and *in vitro* dissolution samples compatible for LC-MS Methods.

Hence, the present investigation was aimed at developing a validated RP-HPLC-PDA method for the analysis of Minocycline hydrochloride in bulk, pharmaceutical dosage forms, accurate, precise and economical.

METHOD OPTIMIZATION

Mobile phase optimization was initially carried out with Inertsil ODS Column (150x4.6 mm, 5µ) using HPLC Water:Methanol(50:50). Minocycline hydrochloride peak was not eluted (Fig.2.2). In the next trial change in mobile phase 0.02%V/v/v Formic acid:Acetonitrile (80:20v/v) were tried on the same column, Minocycline hydrochloride eluted with retention time of 3.36 min but peak fronting was observed (Fig.2.3). In next trial, with same column, change in the mobile phase ratio of 0.02%V/v/v Formic acid:Acetonitrile (85:15v/v). Minocycline hydrochloride eluted at 3.50 min, fronting was observed, the peak symmetry was not good and baseline drift was observed (Fig.2.4). In another trail change in ratio 0.02%V/v/v Formic acid:Acetonitrile (90:10v/v) was used. Minocycline hydrochloride peak shape was broad and peak fronting was observed (Fig.2.5). In the next trial change in mobile phase composition 0.02%V/v/v Formic acid:Methanol (70:30v/v), Minocycline hydrochloride peak eluted at 4.17 min, in this trial tailing factor was

below 2, fronting was not seen and the peak symmetry was good (Fig.2.6). For quantitative analytical purpose wavelength was set at 220 nm, which provided better reproducibility with minimum or no interference. The method was validated as per ICH Guidelines. The peak purity index of Minocycline hydrochloride was found to be greater than 0.9999 indicating peak purity of the drug sample used in the analysis and shown in (Figure-3.1).

The optimized chromatographic conditions were as follows:

Mobile phase	0.02%V/v/v Formic acid:Methanol (70:30v/v)
Flow rate	1.0 mL/min
Mode of elution	Isocratic
Column	Inertsil ODS Column (150x4.6 mm, 5µ)
Detector wave length	220 nm
Column temperature	Ambient
Injection volume	10µL
Run time	7 min
Diluent	Water

With the above developed conditions using 0.02%V/v/v Formic acid:Methanol (70:30v/v) as mobile phase with isocratic mode of elution program at 1.0mL/min flow rate, the method was validated as per ICH guidelines.

Assay/Analysis of the marketed formulation

20 tablets were weighed individually and finely powdered. A powder blend equivalent to 10 mg of minocycline hydrochloride was transferred to a 10mL volumetric flask containing about 5mL of methanol, sonicated and made up to the mark with the same. The resulting solution was filtered through 0.45µm nylon membrane filter to obtain a stock solution of 1mg/mL. It was further diluted with diluent to get the required concentration (30µg/mL of minocycline hydrochloride). The solution was injected three times into the column. From the peak area obtained,

the content of minocycline hydrochloride in the tablets was calculated from the formula given in assay in method validation. The results were given in Table 3.8 and shown in Figure 3.48-3.50. The assay was found to be within the limits, indicating that the present LC conditions can be used for the assay of minocycline hydrochloride in different commercially available formulations.

Acceptance Criteria:

- The percentage assay for the drug should be 100% ±2.
- % RSD of recoveries for the minocycline hydrochloride should be not more than 2.

Table 3.8 Assay Results (n=3)

Labeled amount (mg)	Amount found (mg) (Mean±SD)	% Assay	% RSD
Minocycline hydrochloride - 50mg	49.7±0.174	99.43	0.237

METHOD VALIDATION

An integral part of analytical method development is validation. Once the method has been developed, it is necessary to evaluate under the conditions expected for real samples before being used for a specific purpose. The following parameters were evaluated.

Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components, which may be expected to be present. Typically, these might include impurities, degradants and matrix etc. The effect of wide range of excipients and other additives usually present in the formulation of Minocycline hydrochloride in the determinations under optimum conditions were investigated.

Diluent, placebo, standard solution and sample solution were analyzed individually as per the method to examine interference. From the base shifted overlay of the chromatograms in Fig. 3.2 and the 3D plots of diluent, standard, placebo and formulation are shown in Fig. 3.3, it can be inferred that there were no co eluting or interfering peaks where drug peak eluted. This shows that the peak of analyte was pure and excipients in the formulation did not interfere with the analyte. The peak purity indices values of the standard and sample peaks were found to be greater than 0.9999 and these results were in good agreement with the above results. The peak purity index (1.0000) also confirms the absence of the impurities in pure Minocycline hydrochloride sample. The peak purity index of Minocycline hydrochloride was shown in Fig. 3.1.

Acceptance Criteria:

- Peak purity values should be greater than 0.999.
- Purity angle should be less than purity threshold without having any signs of purity flags.

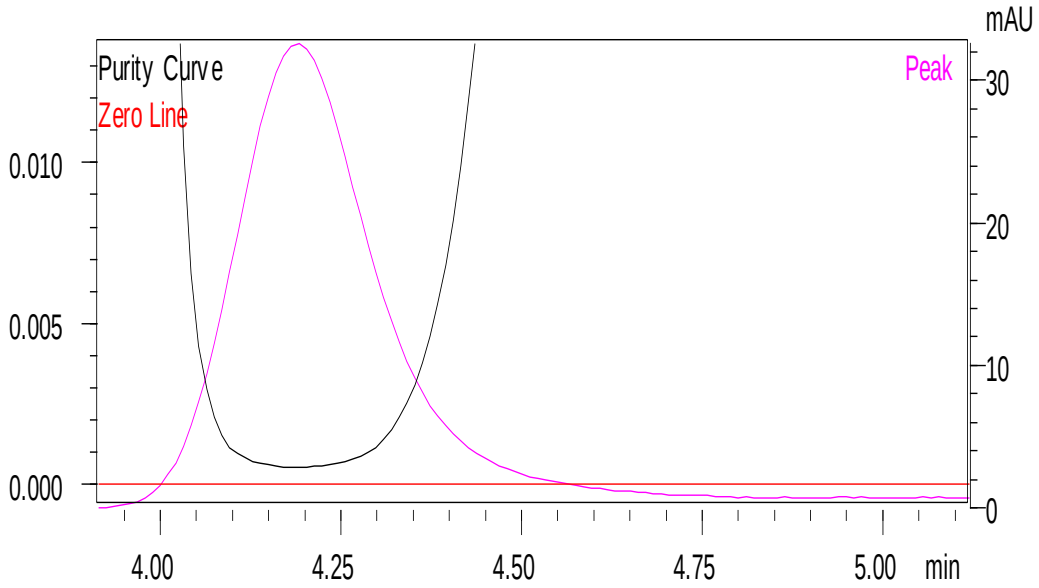


Fig. 3.1 Peak purity index* of Minocycline hydrochloride (* Peak Purity Index:1.000; Single Point Threshold :0.998903; Min.peak purity index:1097).

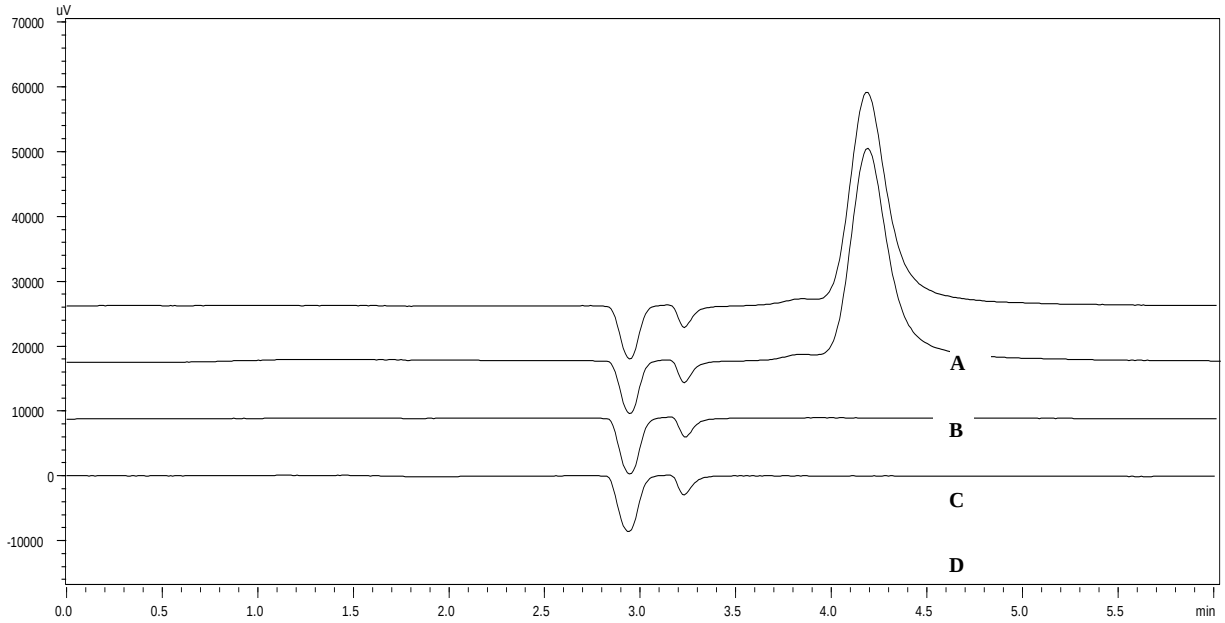


Fig. 3.2 (a) Overlaid base shift chromatograms of A-Standard; B-Sample; C-Placebo ; D-Diluent

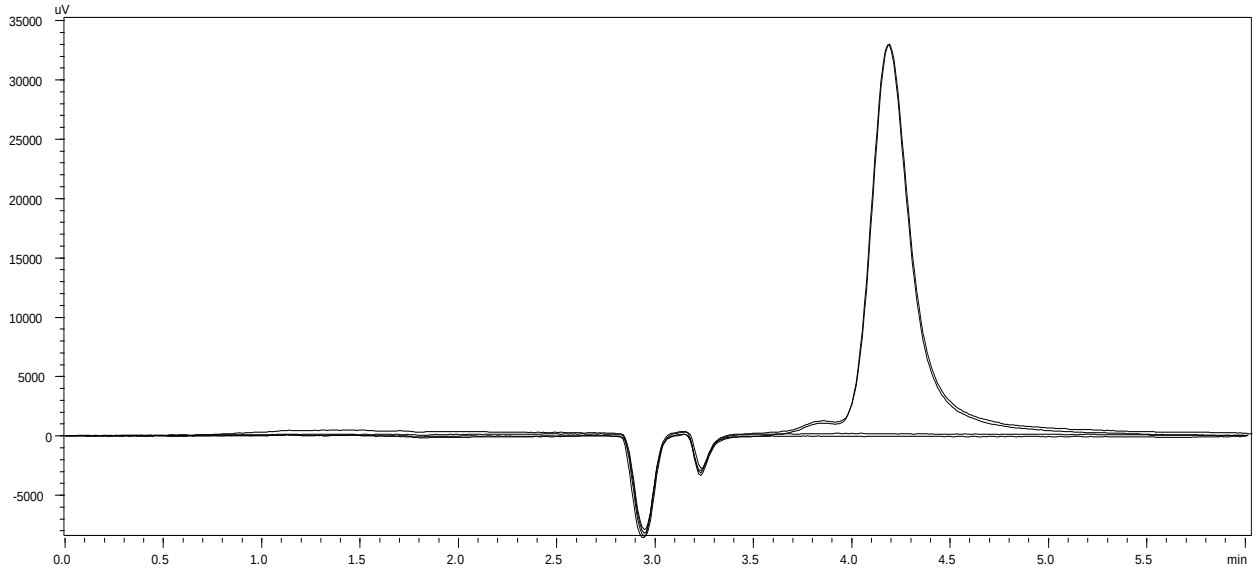
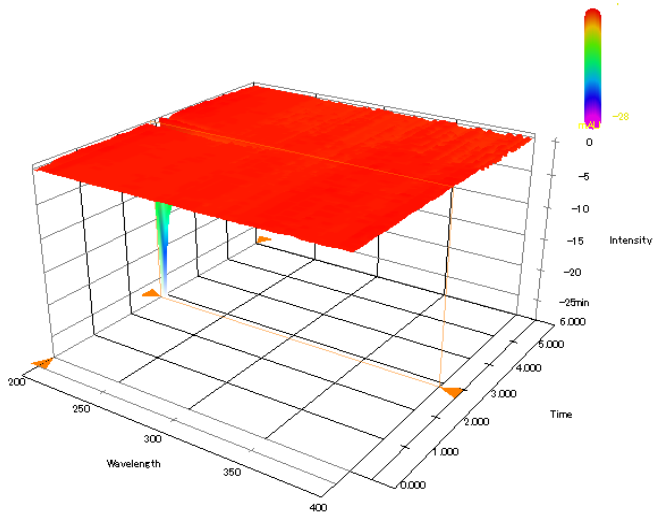
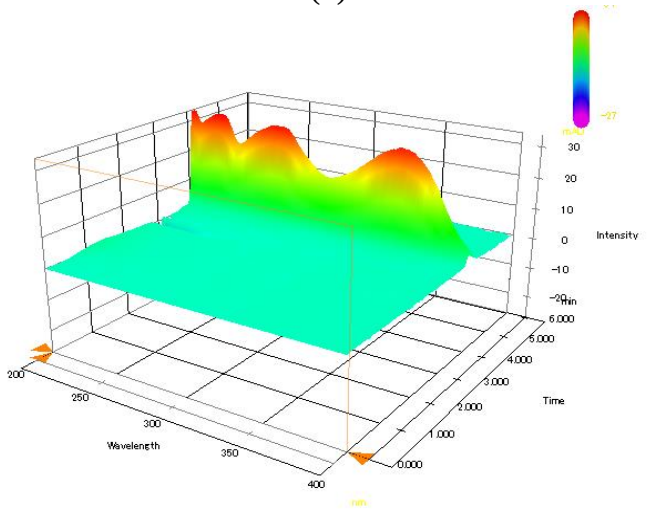


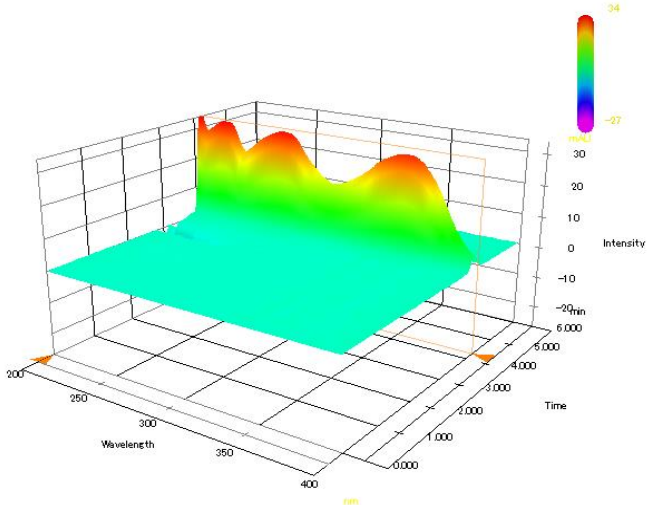
Fig. 3.2 (b) Overlay of the chromatograms of A-Standard; B-Sample; C-Placebo; D-Diluent - without base shift



(1)



(2)



(3)

Fig. 3.3 3D plots of Diluent (1), Placebo (2), Standard (3) and Sample

Linearity

Linearity of an analytical procedure is its ability to obtain test results, which are directly proportional to the concentration (amount) of analyte in the sample. A linear relationship was evaluated across the range of 10-50µg/mL for Minocycline hydrochloride of the analytical procedure in triplicate. The range of concentrations was selected based on 80-120 % of the test concentration. Peak area and concentrations were subjected to least square regression analysis to calculate regression equation. The data of the calibration curve was given in Tables 3.1 and shown in Figures 3.4-3.10.

Acceptance criteria

- The correlation coefficient should be not less than 0.99.
- % RSD should be not more than 2.0.

Table 3.1 Linearity data for Minocycline hydrochloride (n=3)

Concentration (µg/mL)	Mean peak area with ± SD (%RSD)
10	64979± 1042.599 (0.46)
20	124765.3 ± 1054.358 (0.235)
30	189453± 9008.116 (0.061)
40	257763.3± 6810.138 (0.0287)
50	325527.3 ±8679 .238 (0.124)
REGRESSION EQUATION	y = 13082x – 3730
R	0.999
R^2	0.999

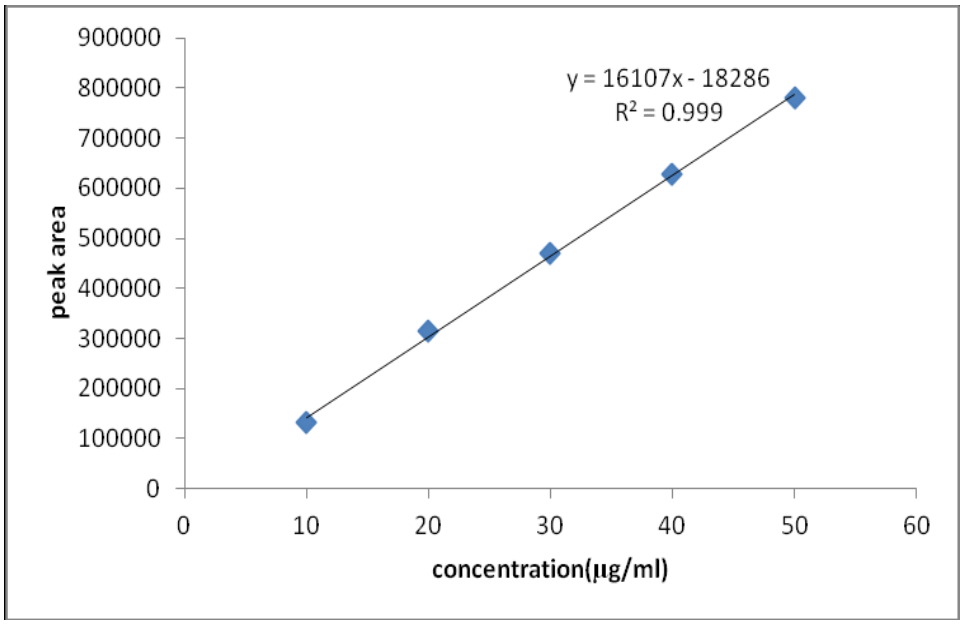


Fig. 3.4 Calibration curve for Minocycline hydrochloride

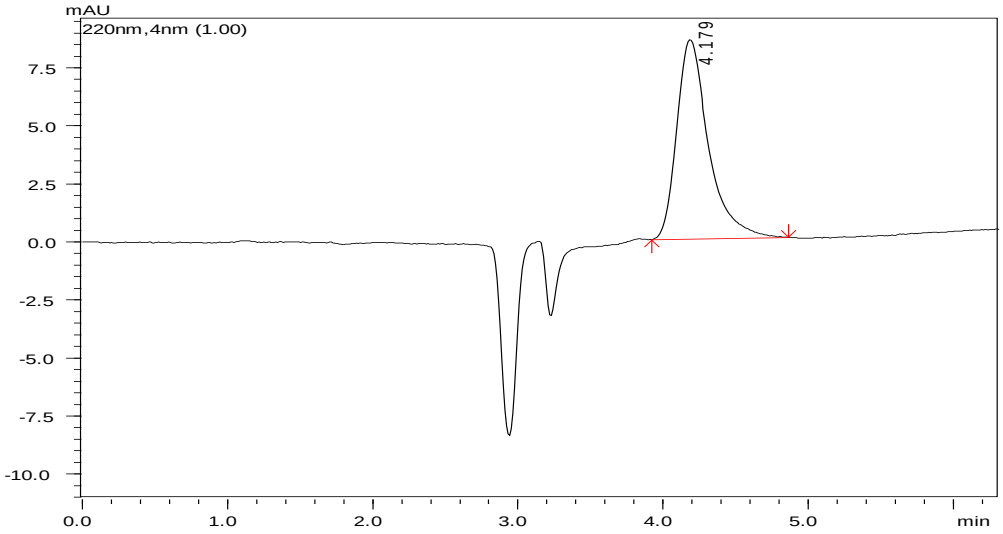


Fig. 3.5 Linearity chromatogram of standard 10µg/mL of minocycline hydrochloride

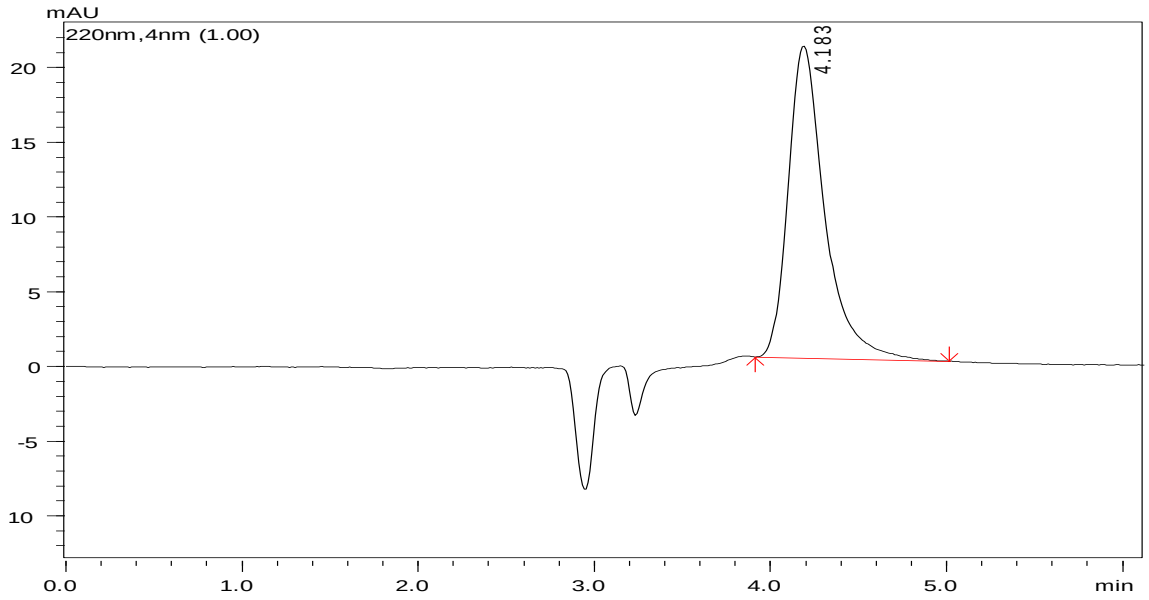


Fig. 3.6 Linearity chromatogram of standard 20µg/mL of minocycline hydrochloride

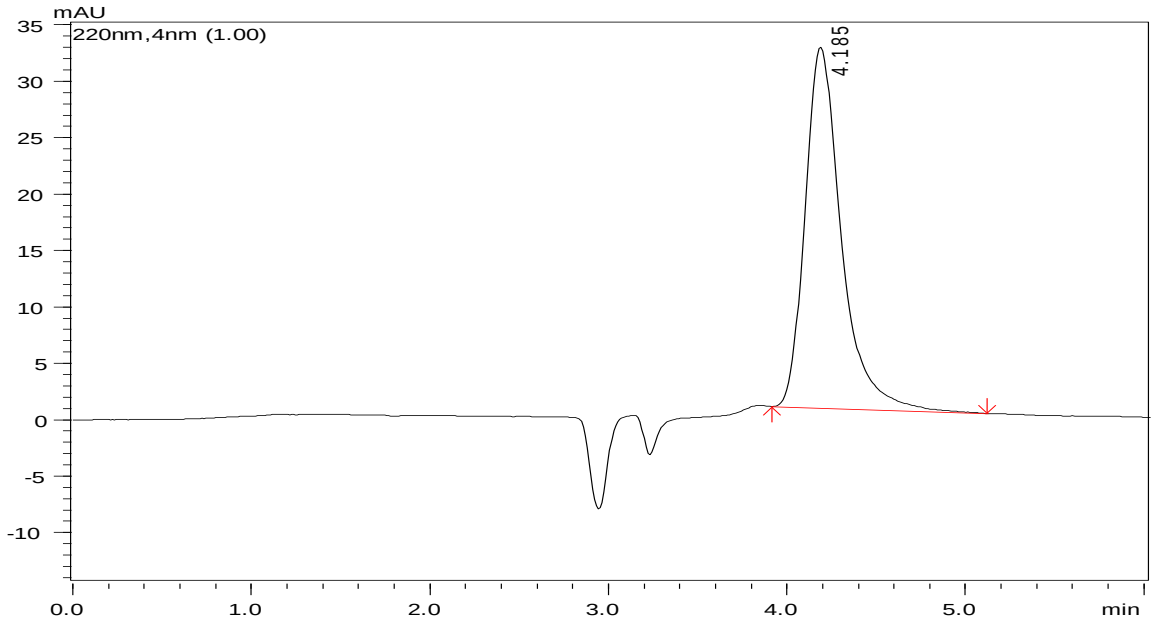


Fig. 3.7 Linearity chromatogram of standard 30µg/mL of minocycline hydrochloride

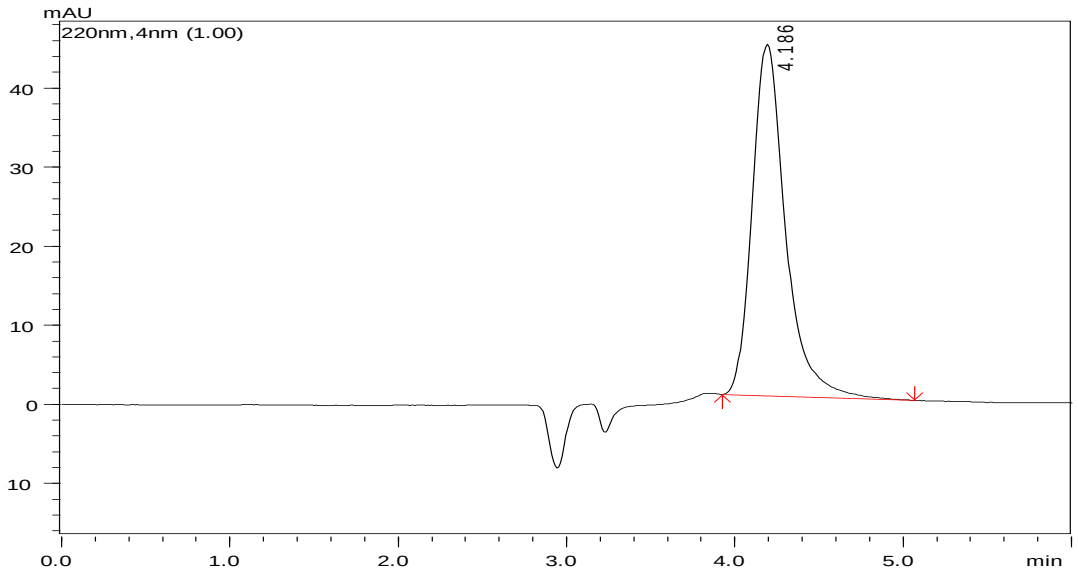


Fig. 3.8 Linearity chromatogram of standard 40µg/mL

minocycline hydrochloride

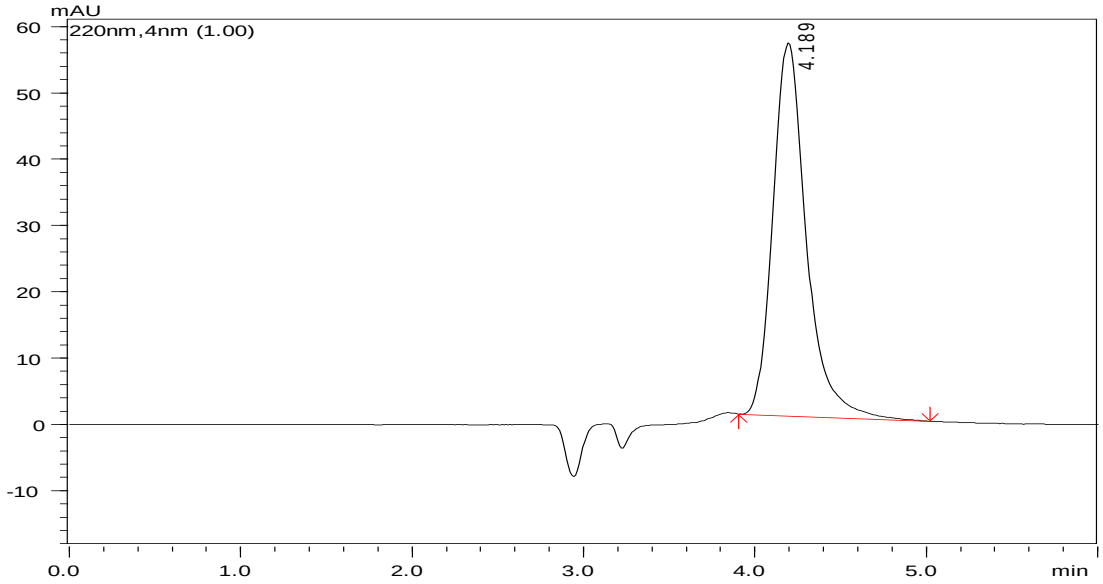
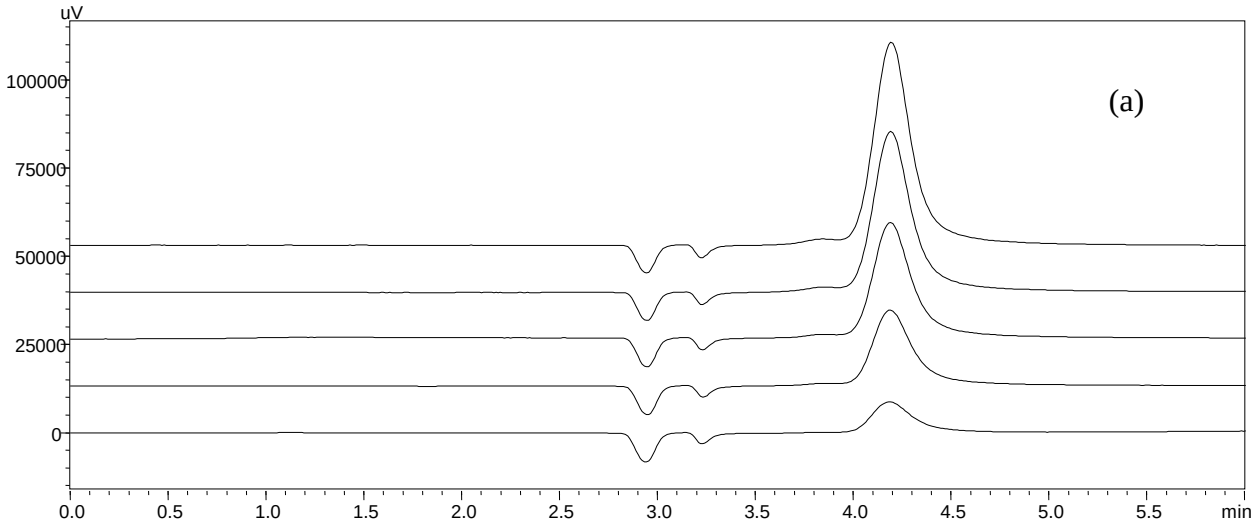


Fig. 3.9 Linearity chromatogram of standard 50µg/mL of minocycline hydrochloride



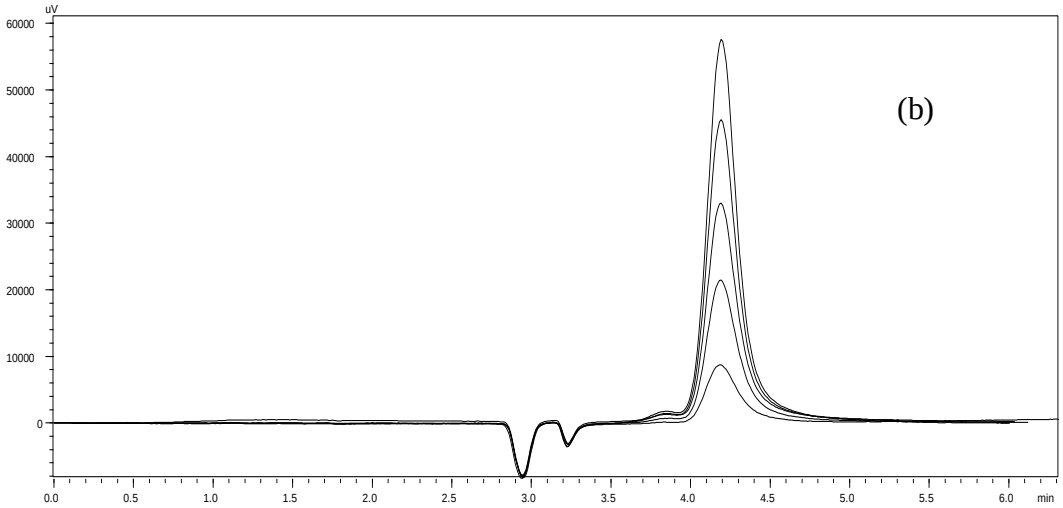


Fig. 3.10 Overlay of chromatograms of standard solution of minocycline hydrochloride at different concentrations (10-50 µg/mL of minocycline hydrochloride) (a)with base shift (b)without base shift

Observation

The correlation coefficient and % RSD were found to be within the limits.

Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed

conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility. Precision should be investigated using homogeneous, authentic samples. The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements. Precision was measured in terms of repeatability of application (System precision) and measurement (Method precision). The precision of the method was ascertained from the peak areas of six replicate injections of a fixed concentration.

System precision:

Repeatability of standard application was carried out using six replicates of the standard concentration 30µg/mL of Minocycline hydrochloride. The data was given in Table 3.2 and chromatograms shown in Figures 3.11-3.17.

Acceptance Criteria:

- % RSD of peak area should not be more than 2.
- % RSD of retention time should not be more than 1.

Table 3.2 System precision data of Minocycline hydrochloride

Injection no.	Peak Area	Retention time
	Minocycline hydrochloride	Minocycline hydrochloride
Injection 1	453254	4.181
Injection 2	452956	4.179
Injection 3	454463	4.180
Injection 4	452806	4.179
Injection 5	454033	4.176
Injection 6	452870	4.175
Average	453397	4.178
Standard deviation	690.332	0.0023
% RSD	0.152	0.055

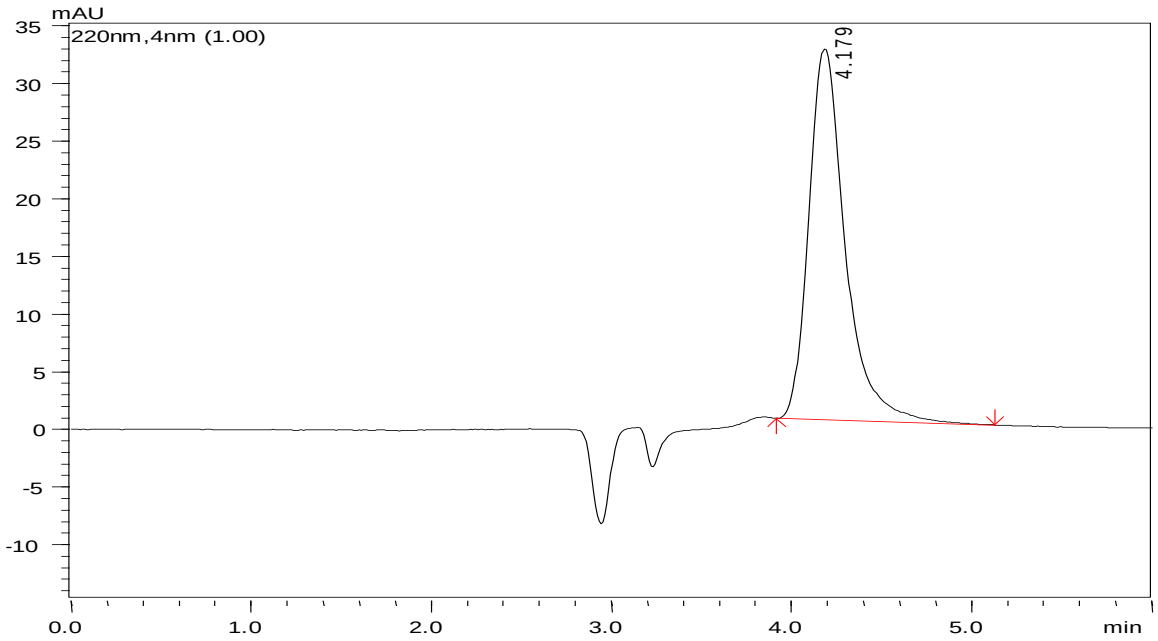


Fig. 3.11 System Precision chromatogram of standard minocycline hydrochloride -Injection 1

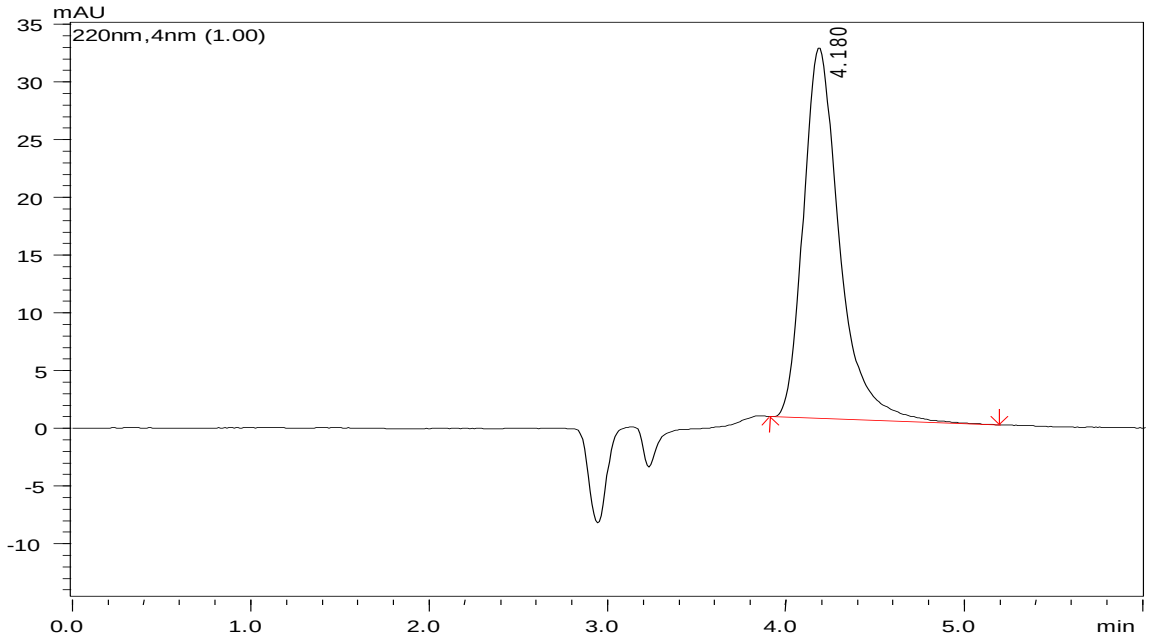


Fig. 3.12 System Precision chromatogram of standard minocycline hydrochloride -Injection 2

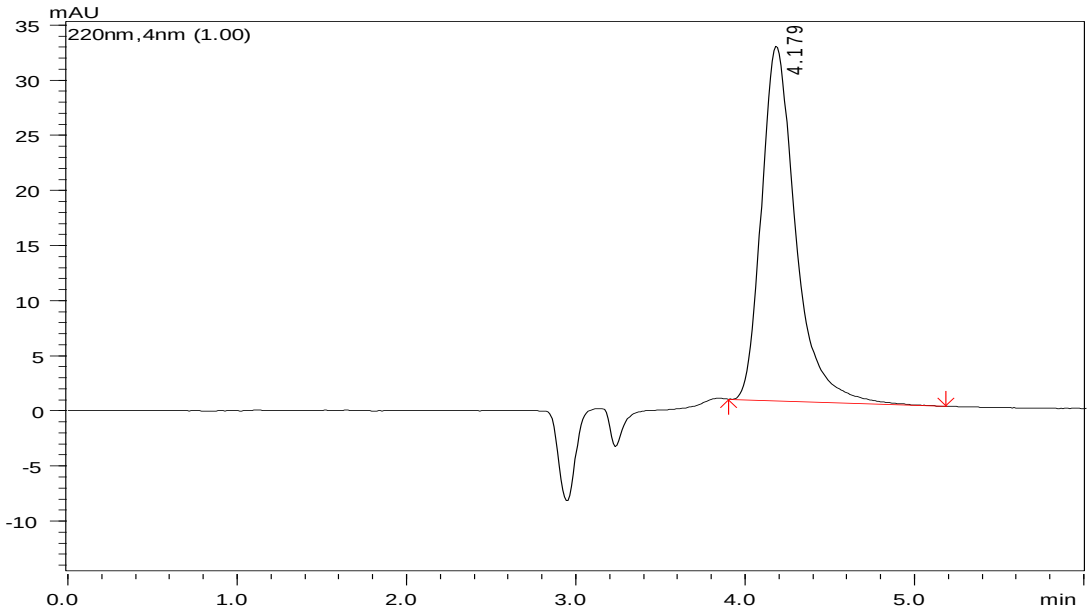


Fig. 3.13 System Precision chromatogram of standard minocycline hydrochloride -Injection 3

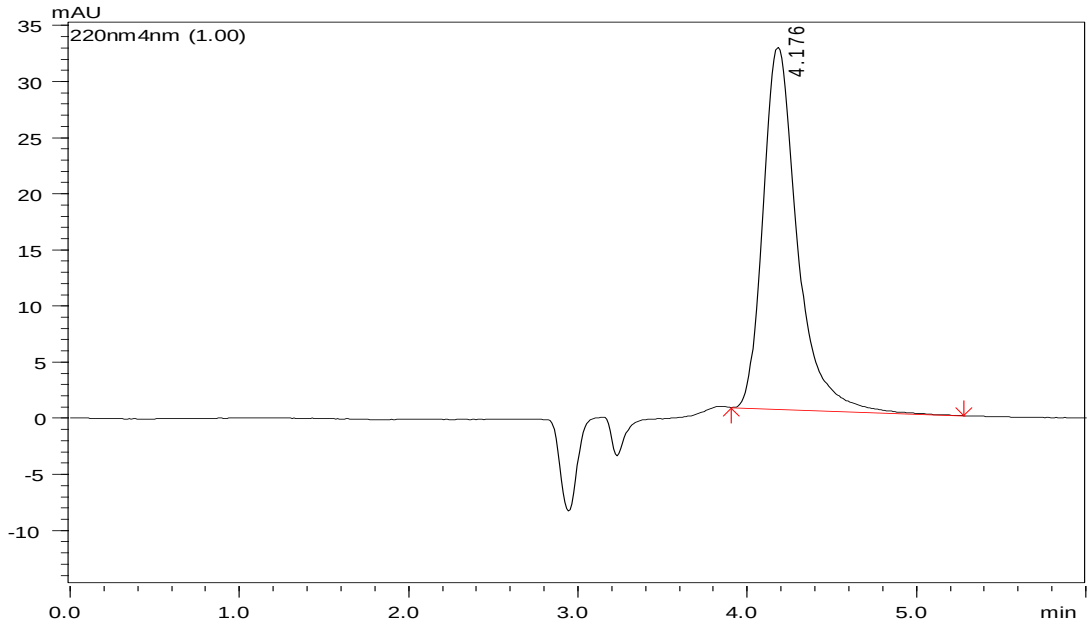


Fig. 3.14 System Precision chromatogram of standard minocycline hydrochloride -Injection 4

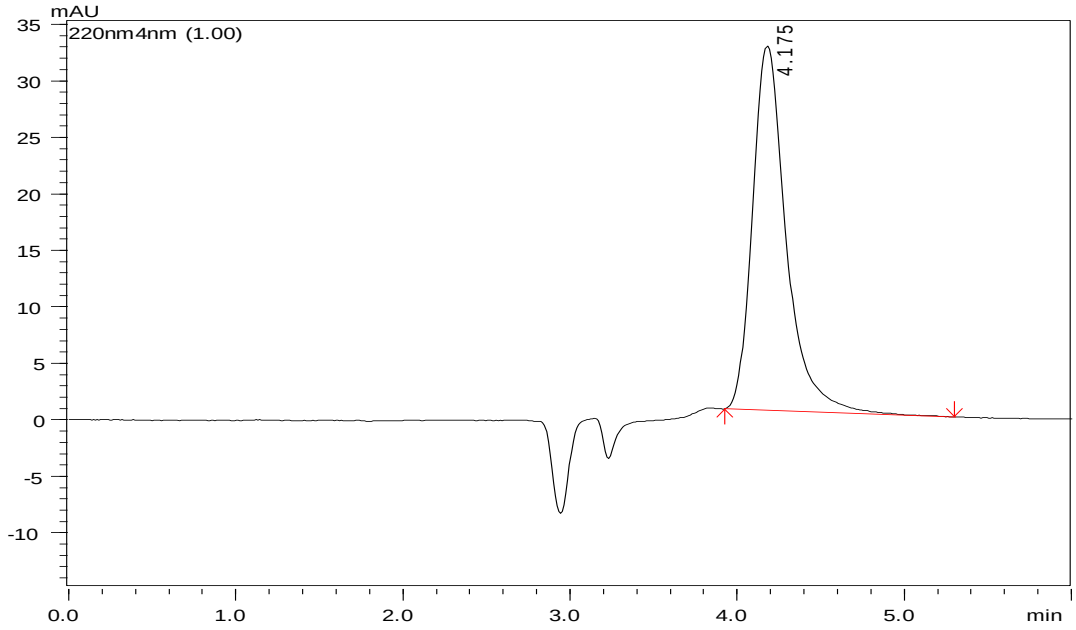


Fig. 3.15 System Precision chromatogram of standard minocycline hydrochloride -Injection 5

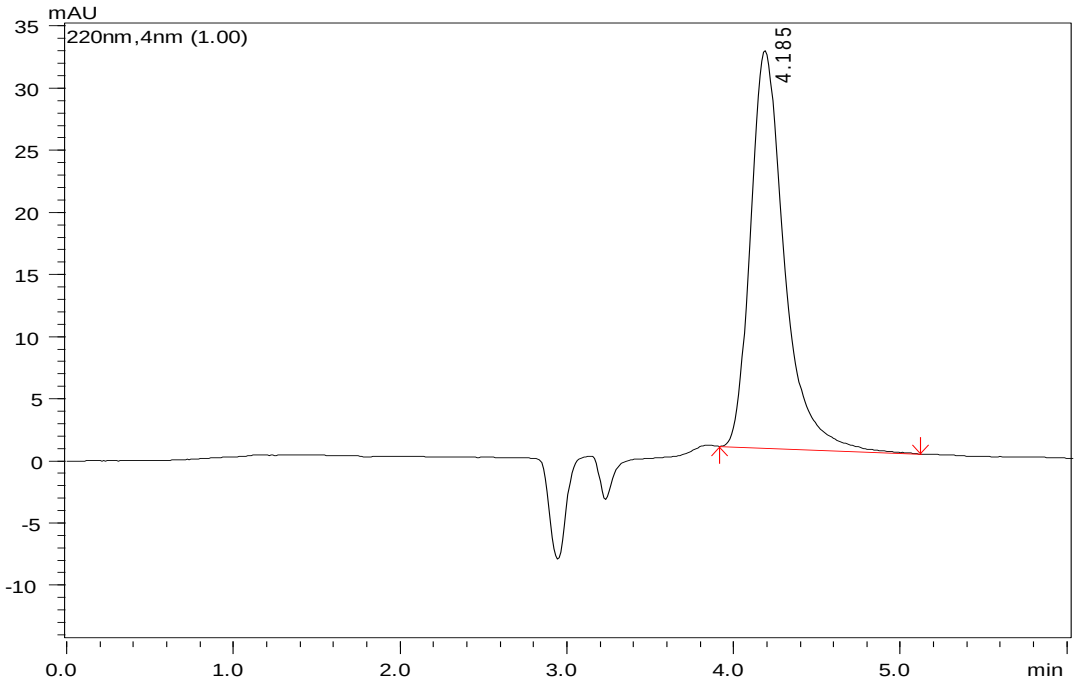


Fig. 3.16 System Precision chromatogram of standard minocycline hydrochloride -Injection 6

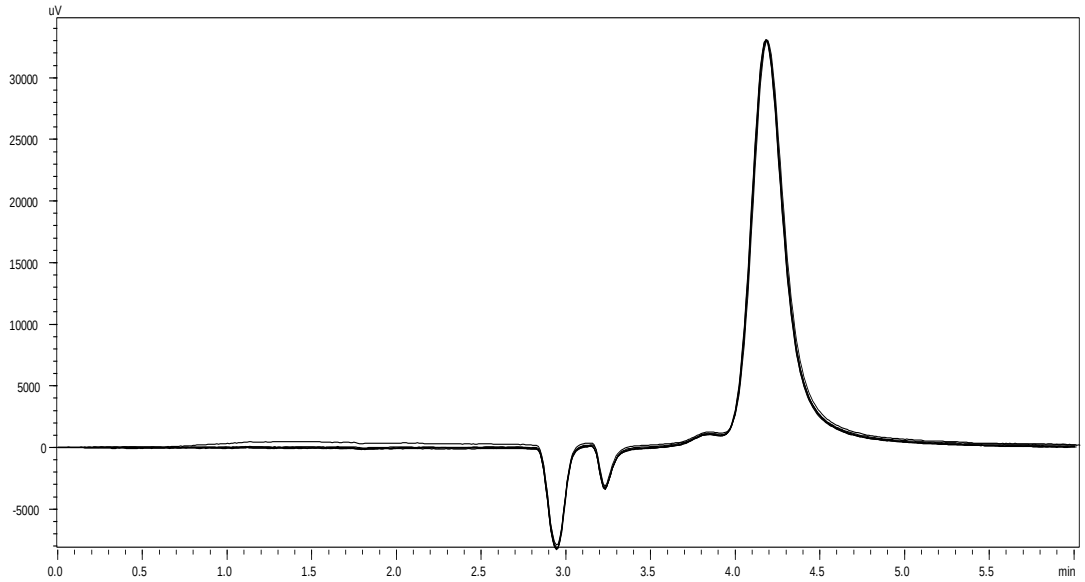


Fig. 3.17 (a) Overlay of system precision chromatograms of standard minocycline hydrochloride

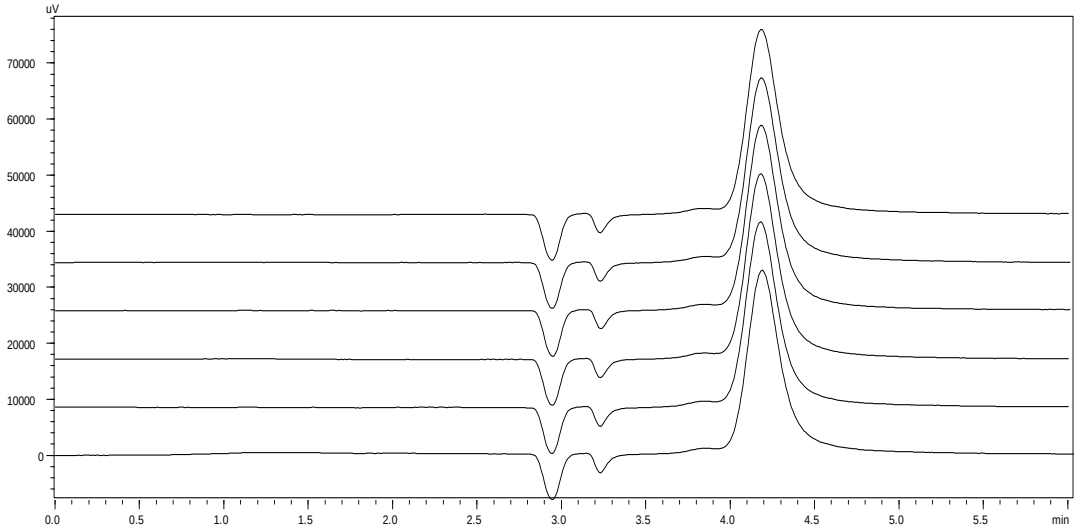


Fig. 3.17 (b) Base shift overlay chromatogram of minocycline hydrochloride

System precision data

Observation

The % RSD was found to be below 2 for areas and below 1 for retention times and therefore fulfilled the ICH guidelines criteria.

Method precision:

Repeatability of sample measurement was carried out in six replicates of the same sample preparations from same homogenous blend of marketed formulation. The data was given in Table 3.3 and chromatograms shown in Figures 3.18-3.24

Acceptance Criteria:

- % RSD of peak area should not be more than 2.
- % RSD of retention time should not be more than 1.

Table 3.3 Method precision data of Minocycline hydrochloride

Injection no.	Peak Area	Retention time
	Minocycline hydrochloride	Minocycline hydrochloride
Injection 1	443982	4.178
Injection 2	442764	4.174
Injection 3	446782	4.178
Injection 4	443187	4.173
Injection 5	444041	4.172
Injection 6	443789	4.174
Average	444091	4.1748
Standard deviation	1408.92	0.0025
% RSD	0.3172	0.061

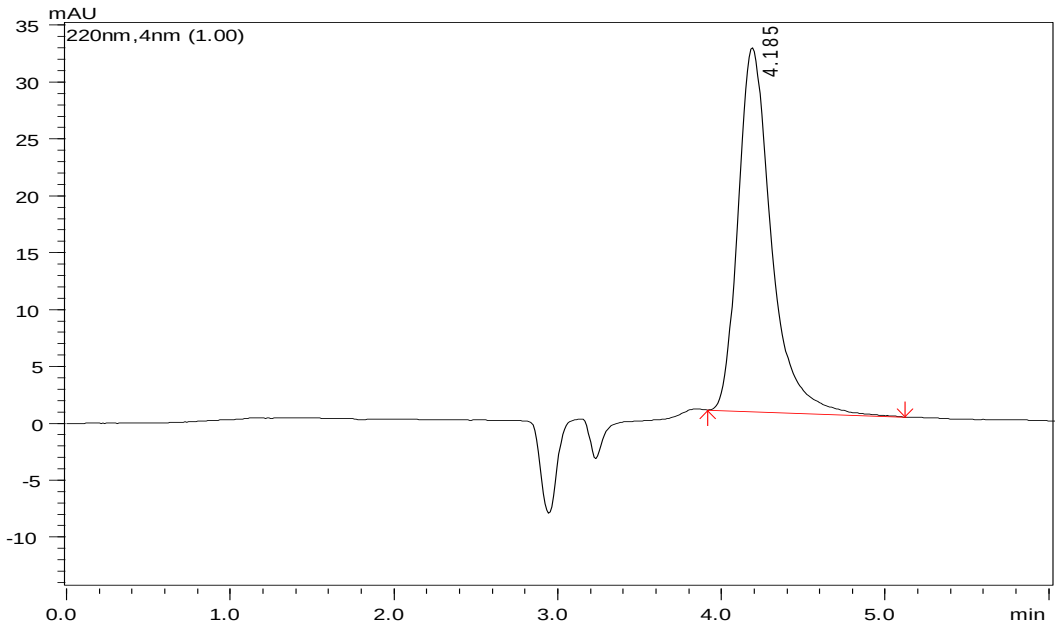


Fig. 3.18 Method Precision chromatogram of minocycline hydrochloride -Injection 1

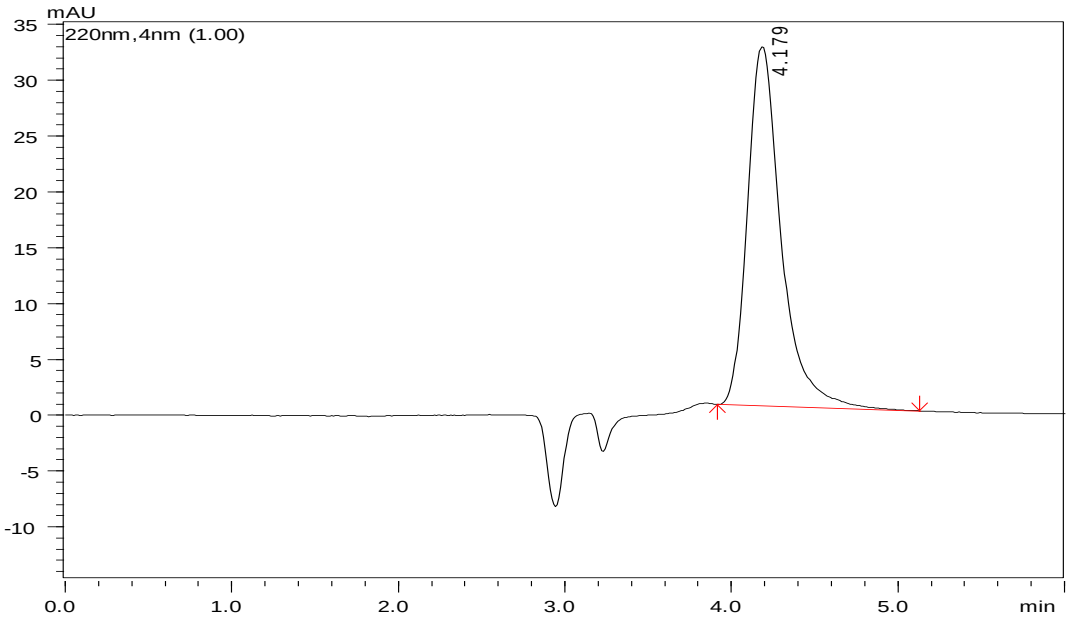


Fig. 3.19 Method Precision chromatogram of minocycline hydrochloride -Injection 2

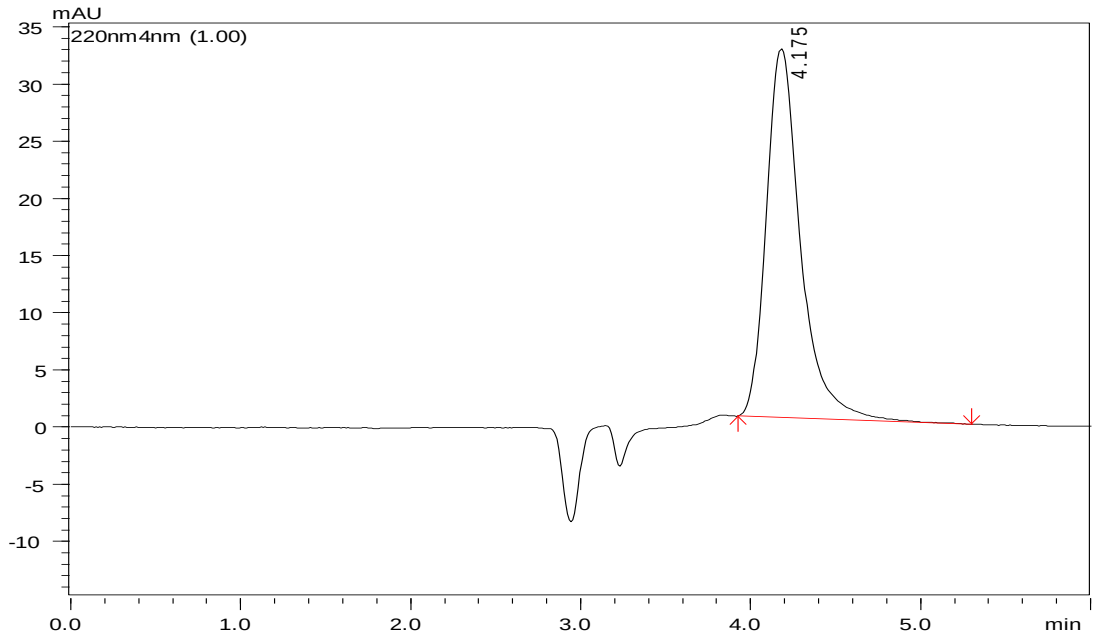


Fig. 3.20 Method Precision chromatogram of minocycline hydrochloride -Injection 3

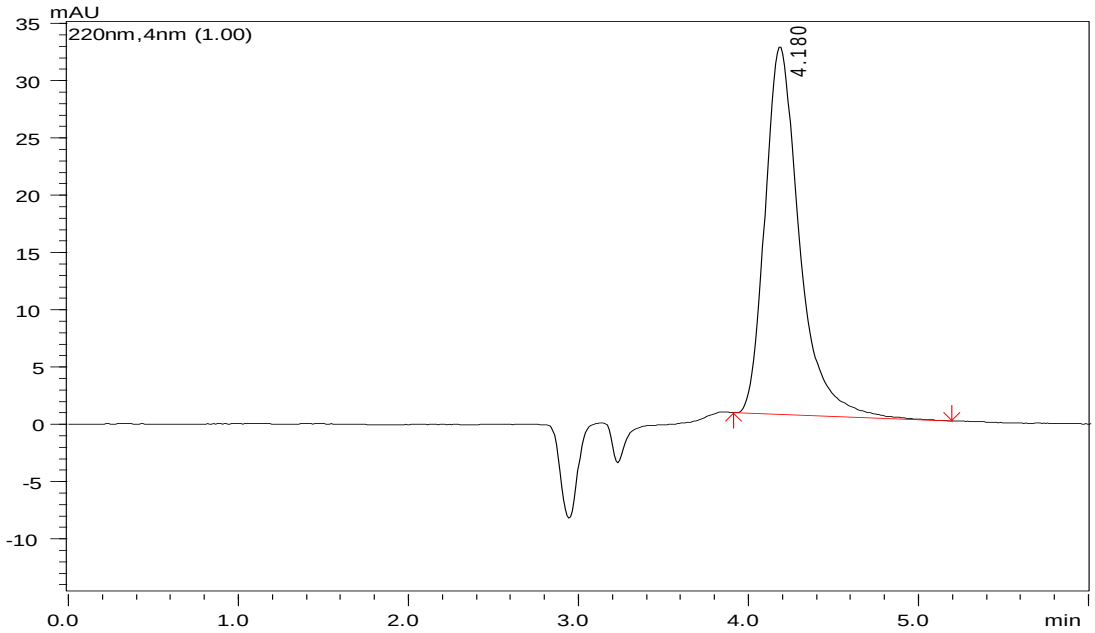


Fig. 3.21 Method Precision chromatogram of minocycline hydrochloride -Injection 4

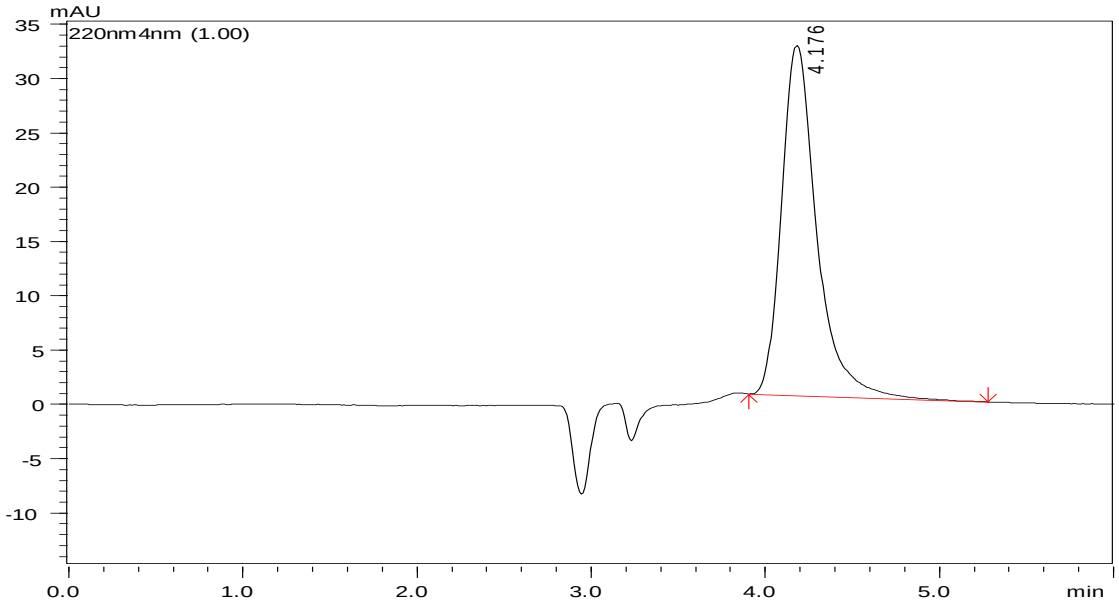


Fig. 3.22 Method Precision chromatogram of minocycline hydrochloride -Injection 5

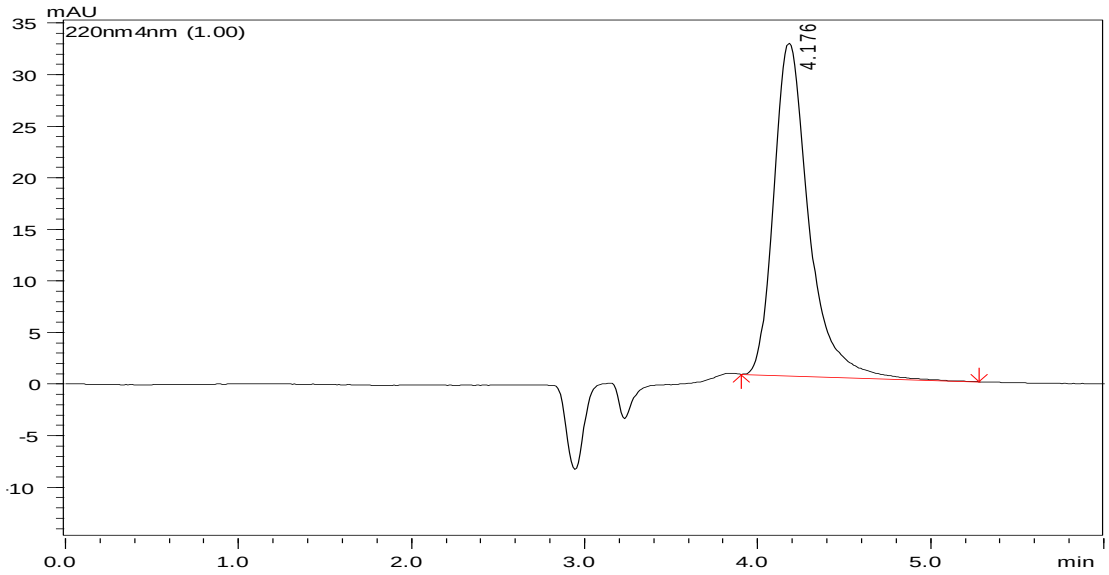


Fig. 3.23 Method Precision chromatogram of minocycline hydrochloride -Injection 6

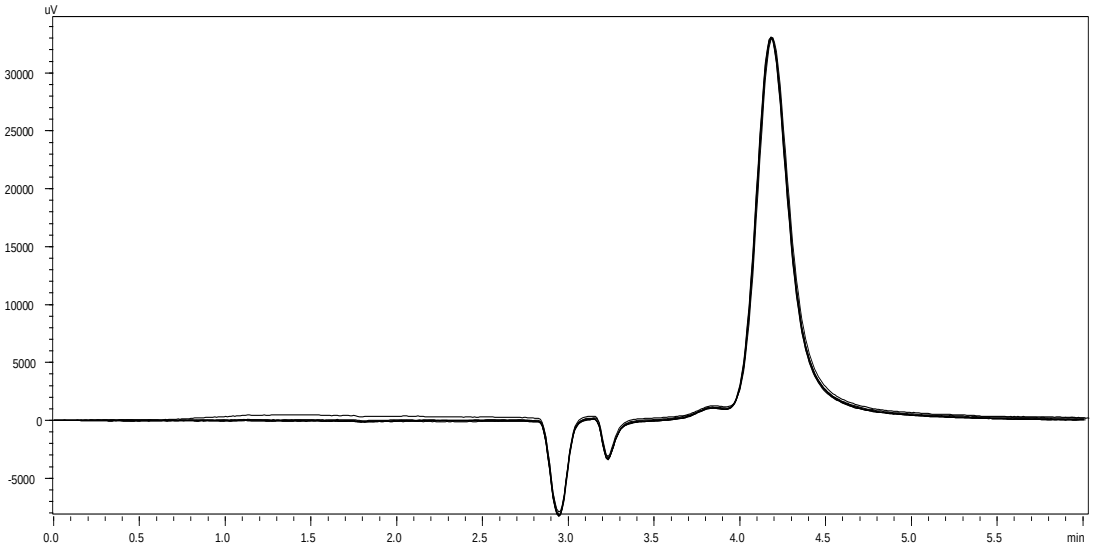


Fig. 3.24 (a) Overlay of method precision chromatograms of minocycline hydrochloride

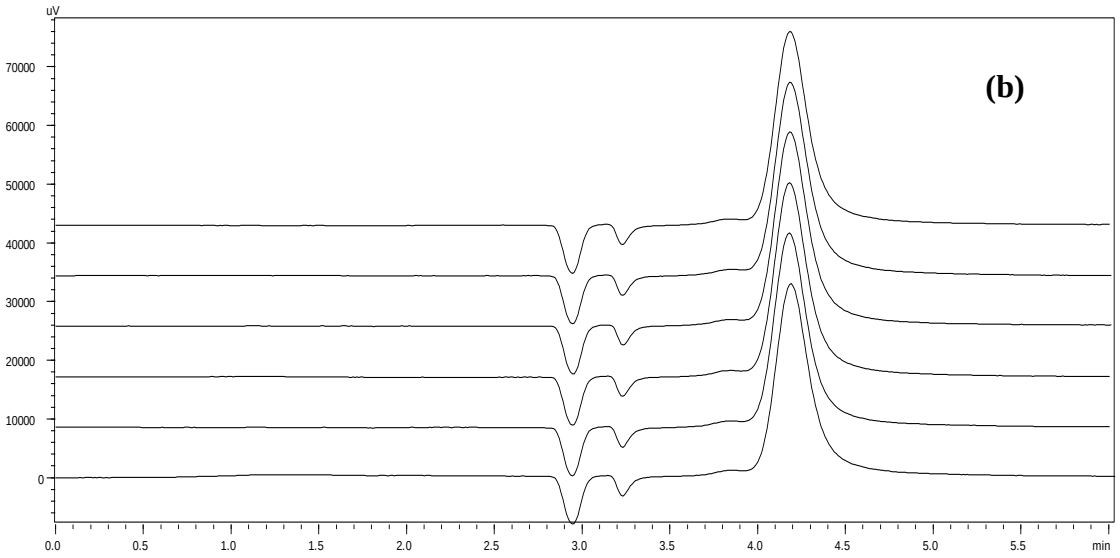


Fig. 3.24 (b) Base shift overlay chromatogram of minocycline hydrochloride Method Precision data

Observation

The % RSD was found to be below 2 for areas and below 1 for retention times and therefore fulfilled the ICH guidelines criteria.

Accuracy/Recovery

The accuracy of an analytical procedure expresses the closeness of

agreement between the value, which is accepted either as a conventional true value or an accepted reference value and the value found. Accuracy was tested (% Recovery and % RSD of individual measurements) by analyzing samples at least in triplicate, at each level (80,100 and 120% of label claim) is recommended.

Accuracy of the method was examined by performing recovery studies by standard addition method for drug product as the exact components are unknown. For the drug substance, the analyte peak was evaluated by 3D plot of the chromatogram in order to confirm the existence of the components at 4.18 elution time of Minocycline Hcl as the impurities were not available. The recovery of the added standard to the drug product sample was calculated and it was found to be 99.52-100.66%. The % RSD was less than 2, which indicate a good accuracy of the method to that of label claim. The obtained recovery results were given in table 3.4. From the 3D plots (in Fig 3.3) it is clear that the peaks eluted at 3.2 min (in standard chromatograms) are only one component, free of impurities. The data was given in Tables 3.4 and shown in Figures 3.25-3.33.

Acceptance criteria:

- The percent recoveries for Minocycline hydrochloride should be in between 98-102.
- % RSD of recoveries for Minocycline hydrochloride should be not more than 2.

Table 3.4 Accuracy data for Minocycline hydrochloride (n=3)

Serial No.	% Level of Addition	Amount Present (µg/mL)	Amount Added (µg/mL)	Amount found (µg/mL)	% Recovery	Mean	%RSD
1	80	30	24	54.22	100.757		
	80	30	24	54.17	100.59	100.523	0.264
	80	30	24	54.07	100.23		
2	100	30	30	59.83	99.45		
	100	30	30	59.98	99.93	99.52	0.372
	100	30	30	59.76	99.20		
3	120	30	36	65.94	99.80		
	120	30	36	66.42	101.40	100.66	0.144
	120	30	36	66.23	100.78		

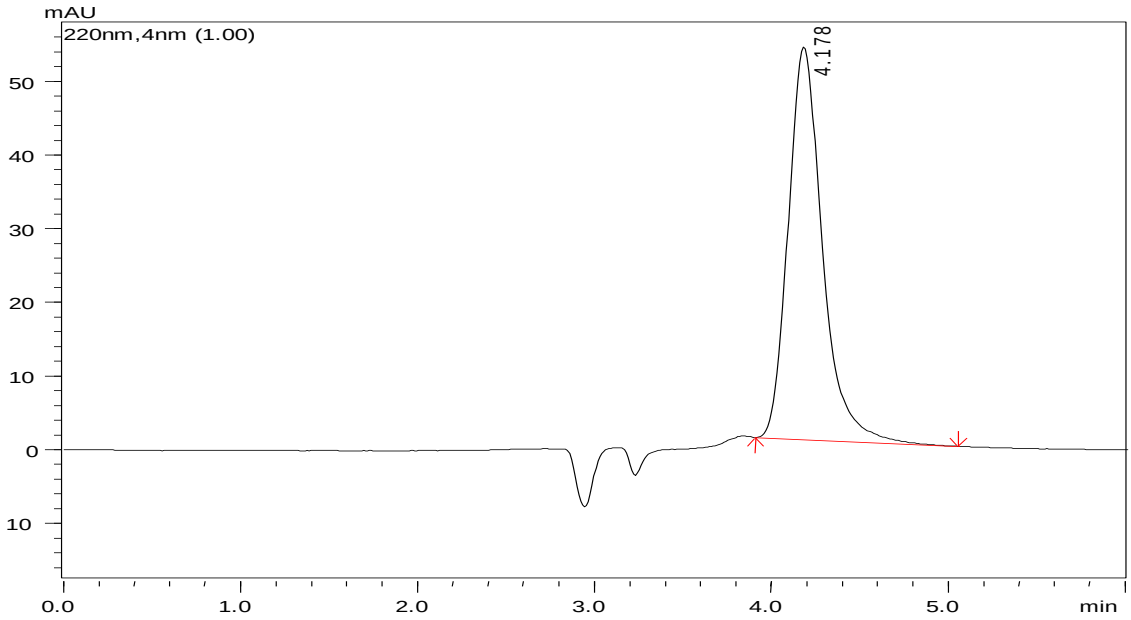


Fig. 3.25 Accuracy chromatogram of minocycline hydrochlorid

at 80%-1 level of addition

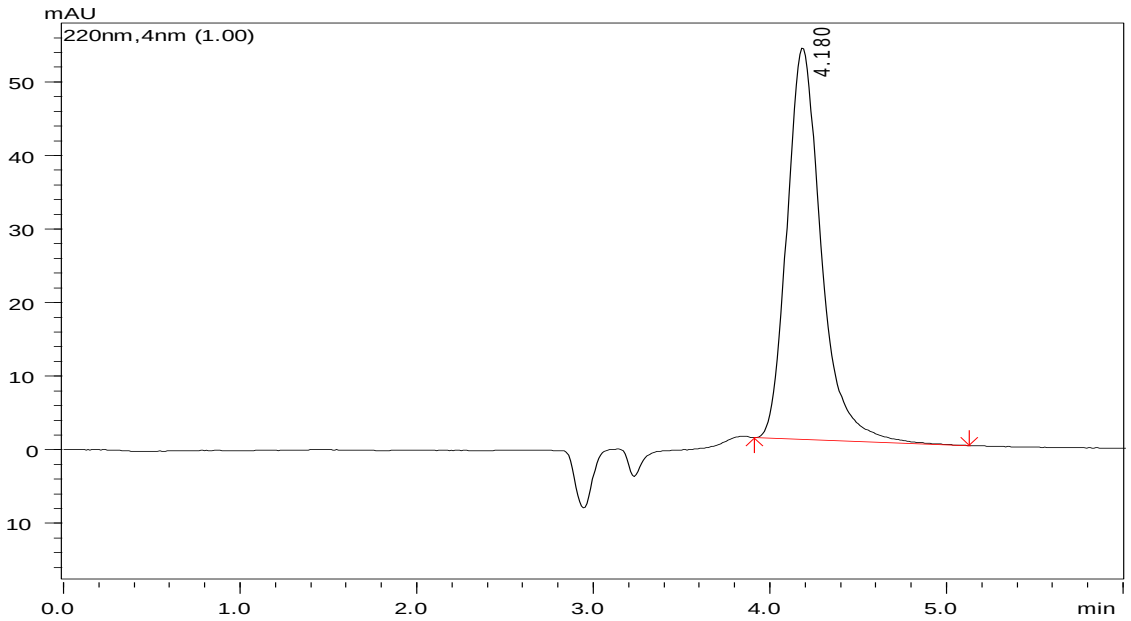


Fig. 3.26 Accuracy chromatogram of minocycline hydrochloride

at 80% -2 level of addition

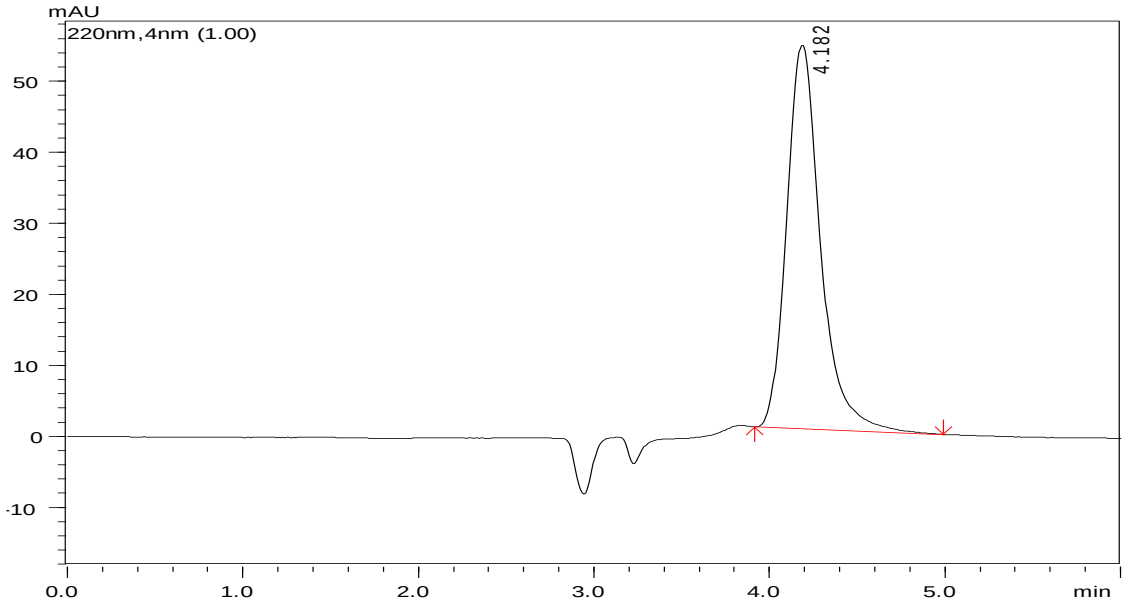


Fig. 3.27 Accuracy chromatogram of minocycline hydrochloride at 80% -3 level of addition

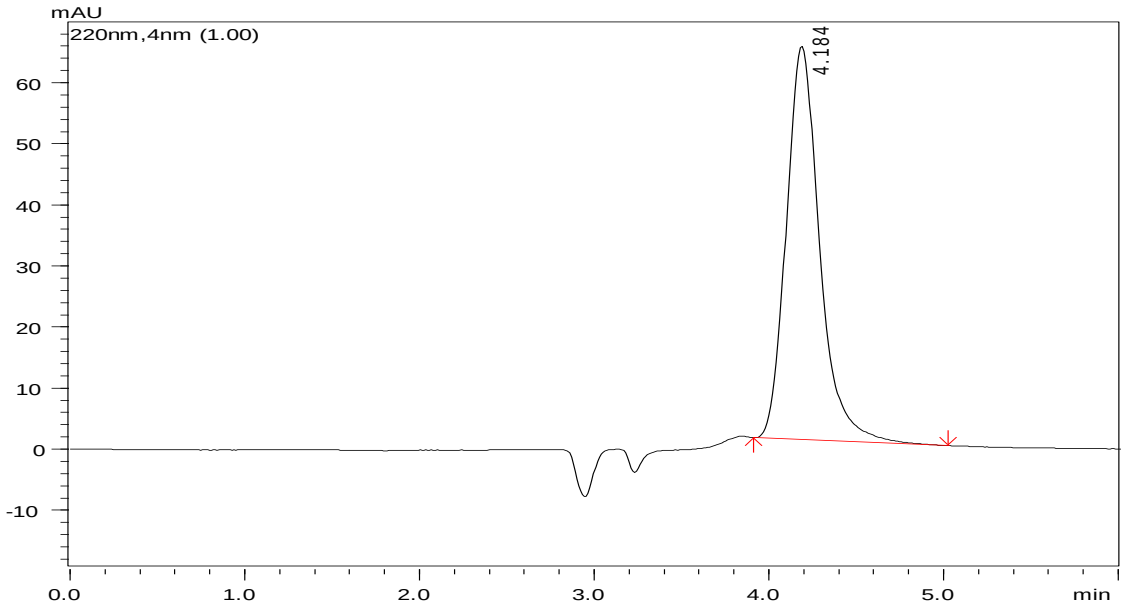


Fig. 3.28 Accuracy chromatogram of minocycline hydrochloride at 100%-1 level of addition

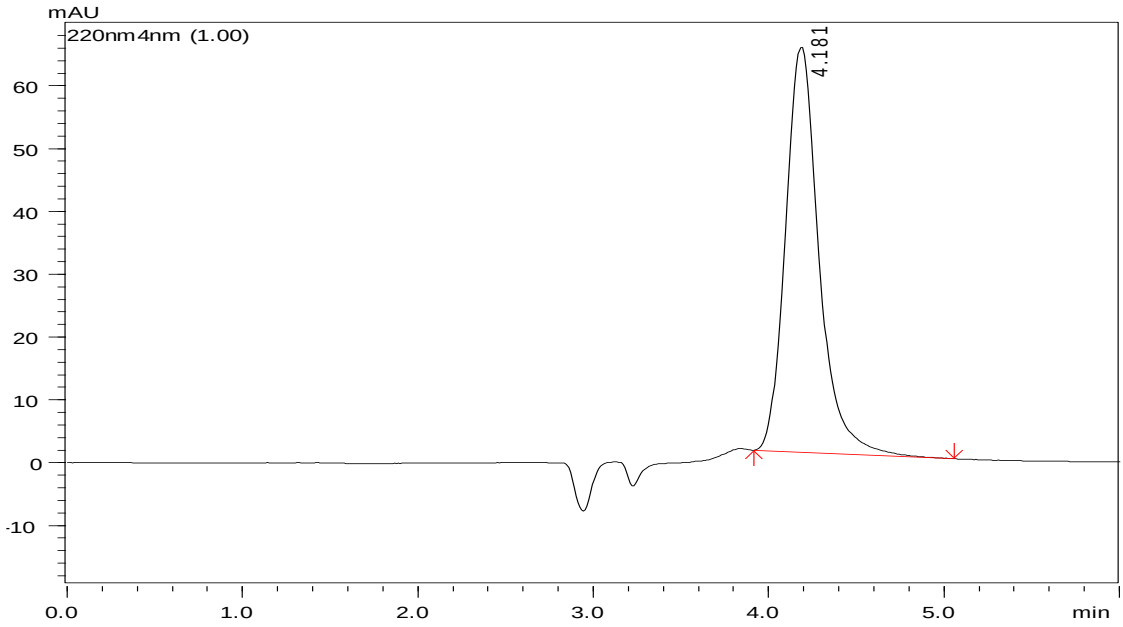


Fig. 3.29 Accuracy chromatogram of minocycline hydrochloride at 100%-2 level of addition

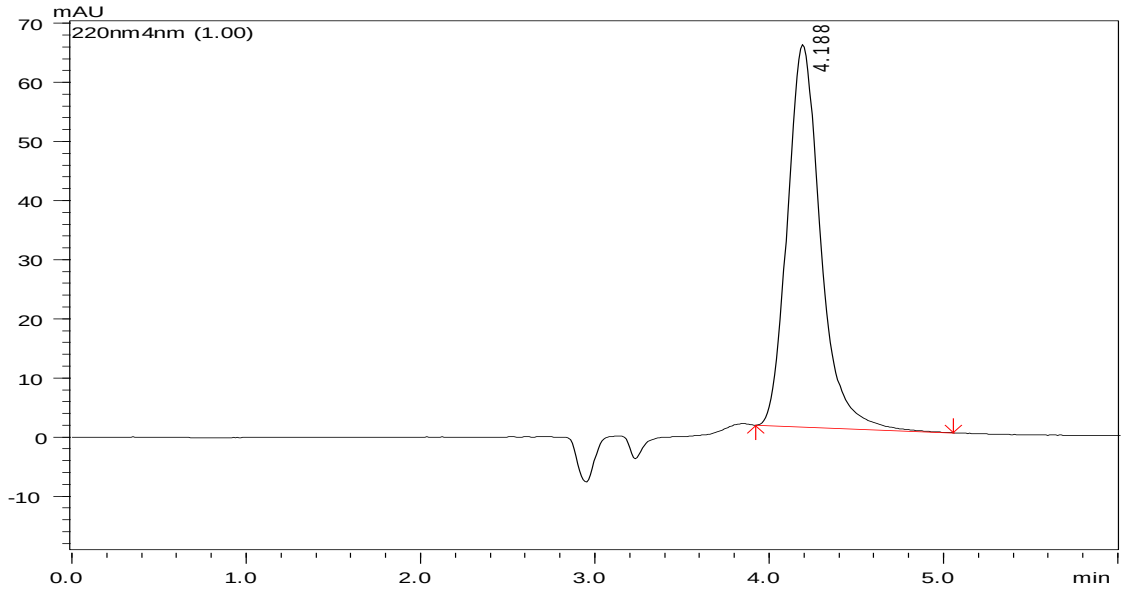


Fig. 3.30 Accuracy chromatogram of minocycline hydrochloride at 100%-3 level of addition

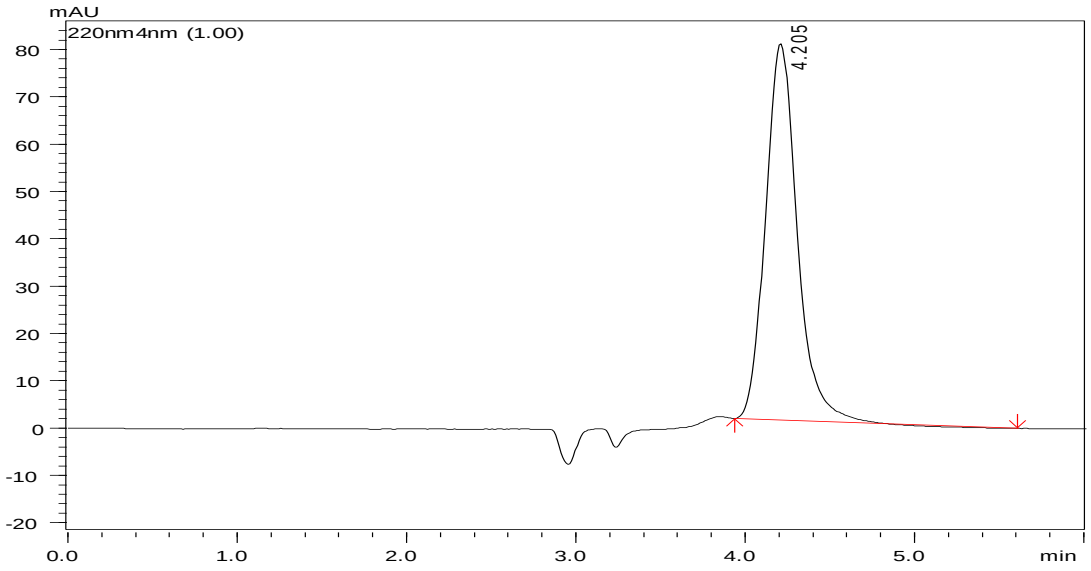


Fig. 3.31 Accuracy chromatogram of minocycline hydrochloride at 120%-1 level of addition

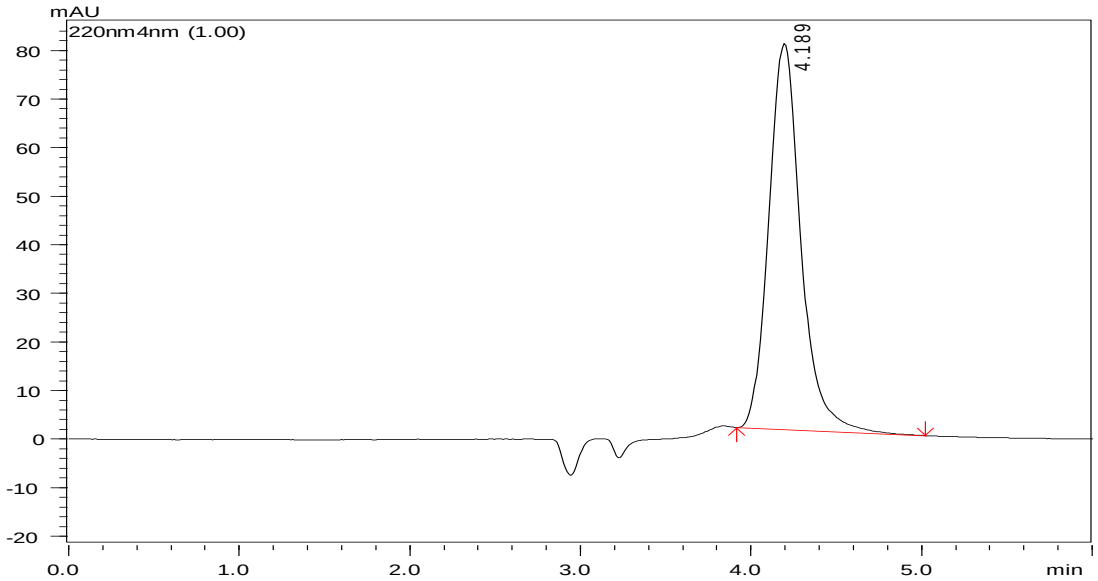


Fig. 3.32 Accuracy chromatogram of minocycline hydrochloride at 120%-2 level of addition

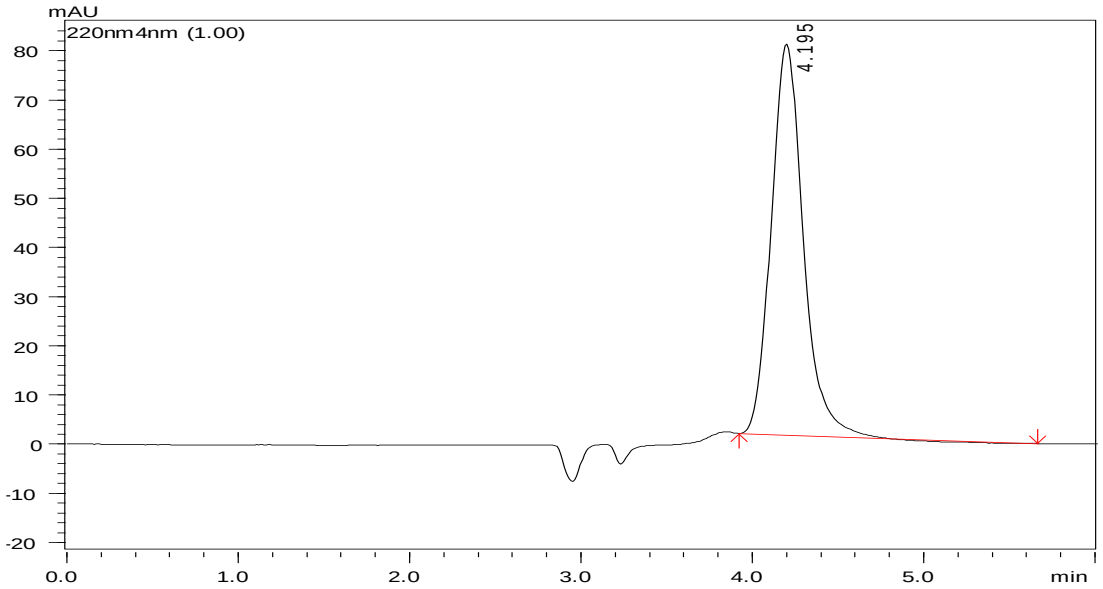


Fig. 3.33 Accuracy chromatogram of minocycline hydrochloride at 120%-3 level of addition

Observation

The obtained percent recoveries and % RSD value of minocycline hydrochloride was found to be within the limits indicating the accuracy of the proposed method.

Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in the method parameters and provides an indication of its reliability during normal usage. In the case of liquid chromatography, examples of typical variations are:

- Influence of variations in mobile phase
- Influence of variations in flow rate
- Influence of variations in wave length

As a part of the robustness, deliberate changes in the flow rate and wavelength were made to evaluate the impact on the method. Changes in the flow

rate slightly affected the retention times of the Minocycline hydrochloride. However the parameters like, theoretical plate number and tailing factor were not changed and were within the limits. Similar results were obtained with the changed wavelength. The data was given in Tables 3.5 and shown Figures 3.34-3.39.

Acceptance criteria:

The robustness should pass as per the test method at variable conditions.

Table 3.5 Robustness data for Minocycline hydrochloride

Chromatographic parameter	Retention time (min)	Theoretical plates (#)	Tailing factor (T_f)
Flow rate			
0.95	4.377	2270.89	1.413
1	4.285	2233.40	1.405
1.05	4.286	2190.75	1.390
Wave length (nm)			
218	4.185	2131.124	1.443
220	4.185	2133.409	1.445
222	4.185	2134.630	1.446

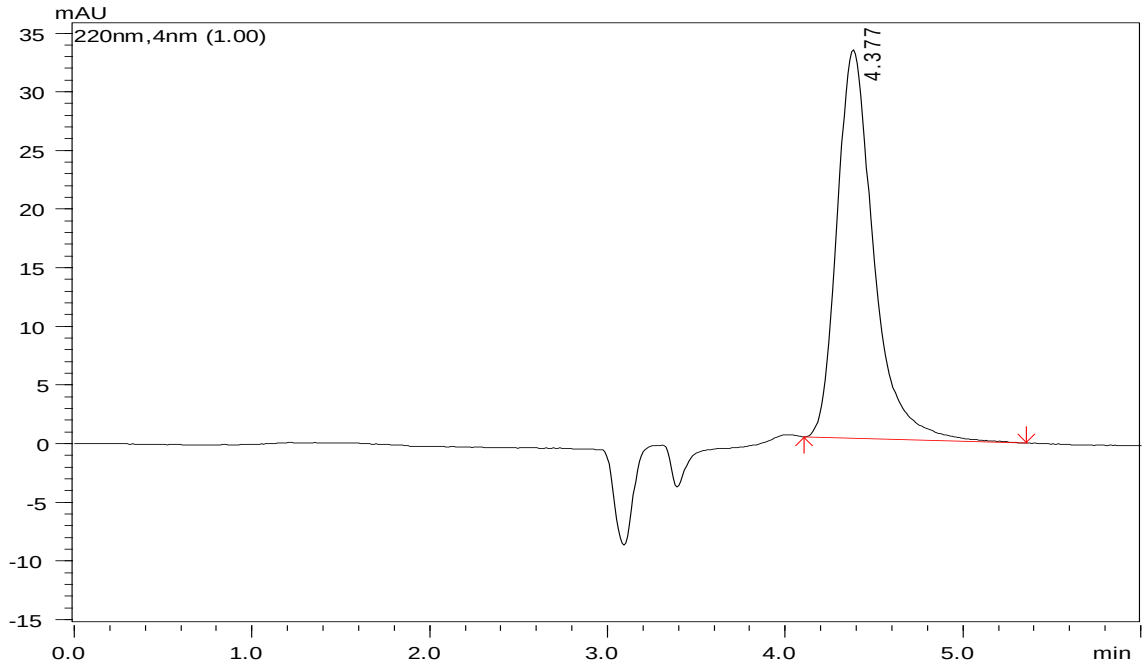


Fig. 3.34 Robustness chromatogram of minocycline hydrochloride at change in flow rate (0.95ml/min)

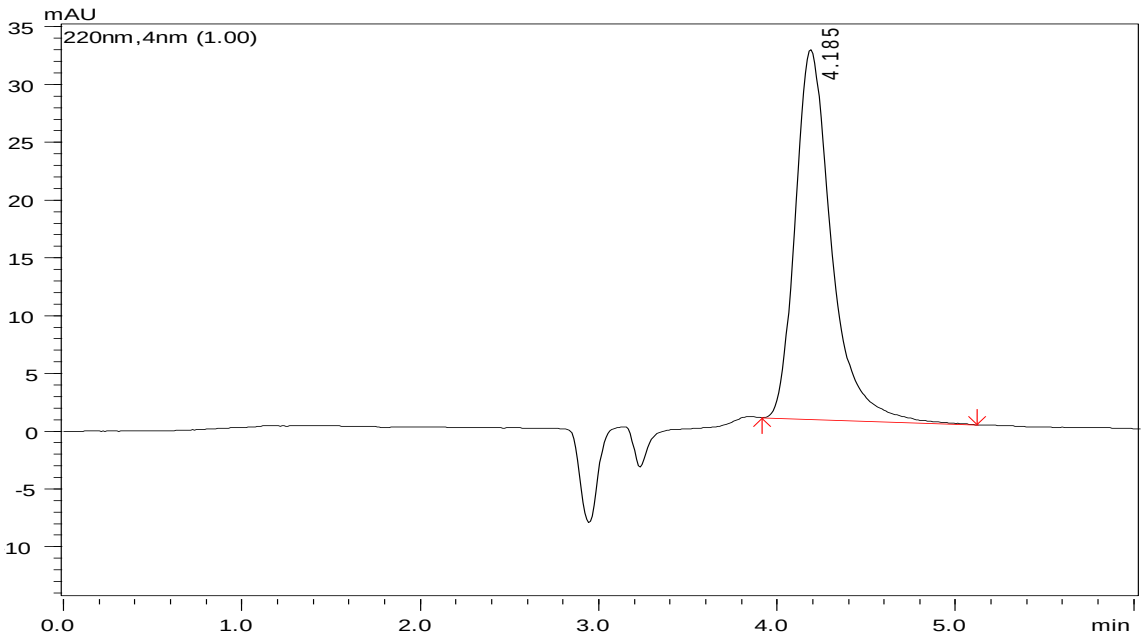


Fig. 3.35 Robustness chromatogram of minocycline hydrochloride at change in flow rate (1 ml/min)

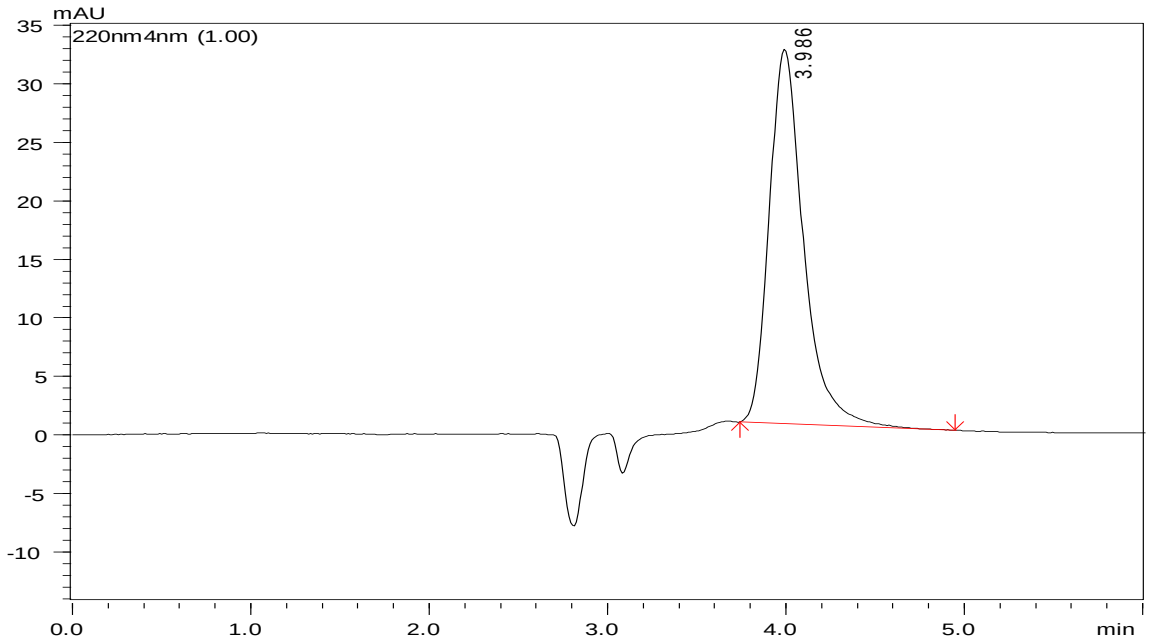


Fig. 3.36 Robustness chromatogram of minocycline hydrochloride at change in flow rate (1.05ml/min)

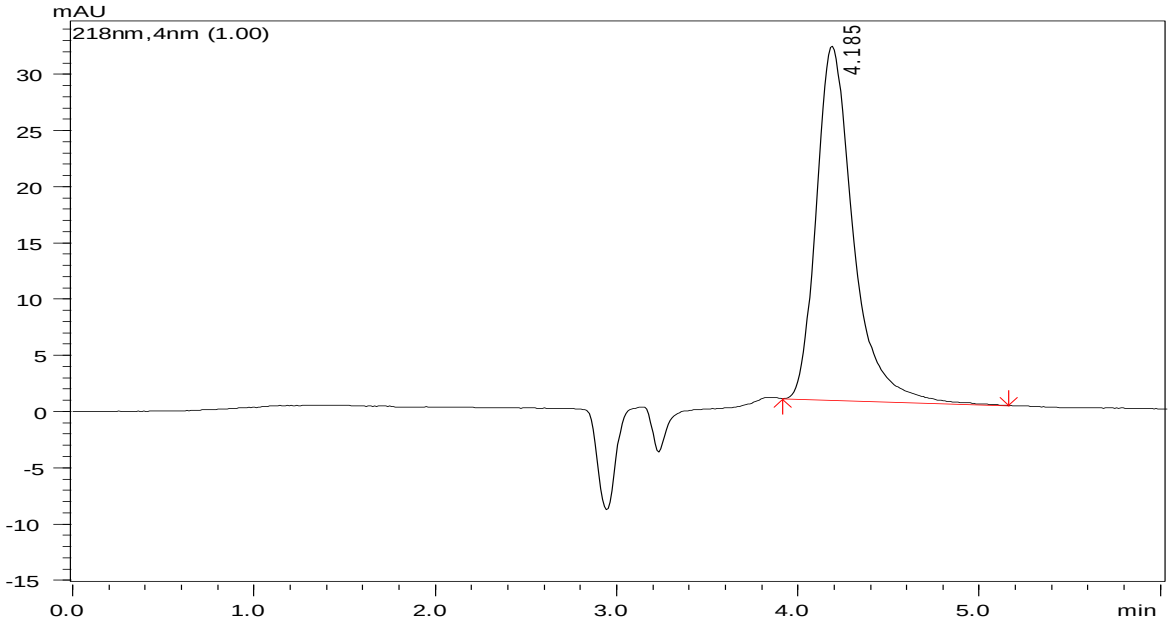


Fig. 3.37 Robustness chromatogram of minocycline hydrochloride at 218nm wave length

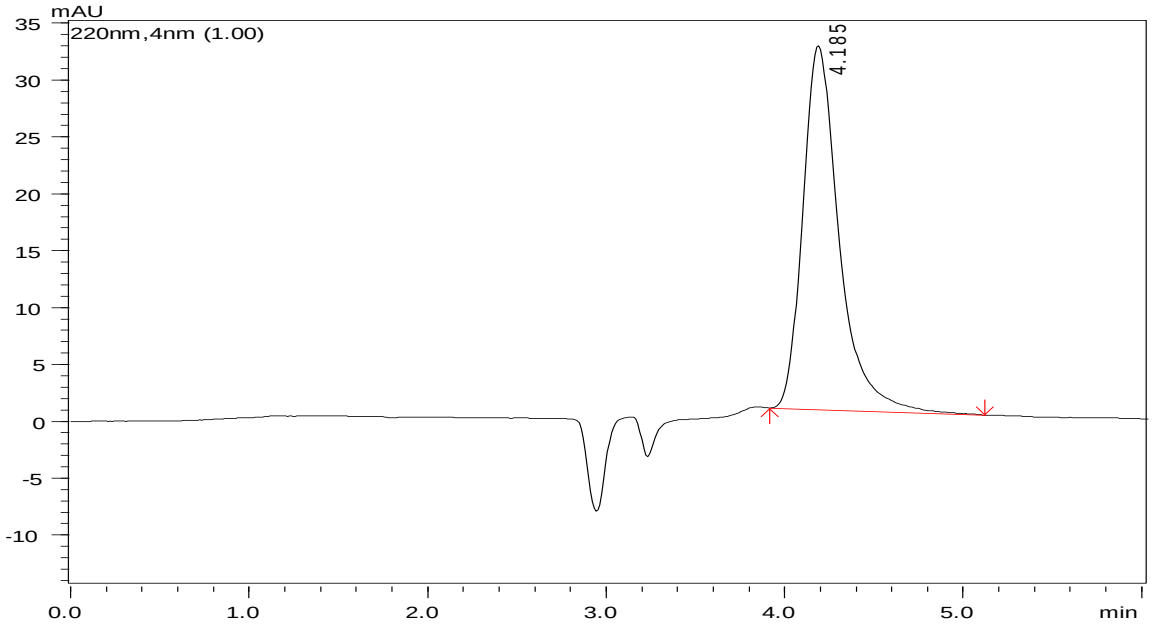


Fig. 3.38 Robustness chromatogram of minocycline hydrochloride at 220nm wave length

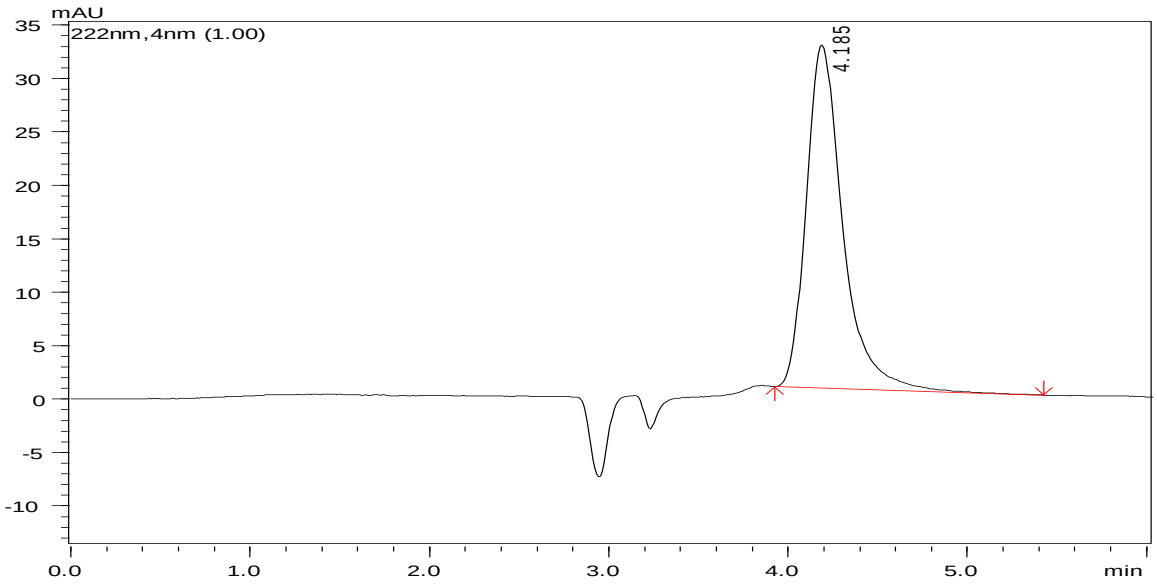


Fig. 3.39 Robustness chromatogram of minocycline hydrochloride at 222nm wave length

Observation

From the above results obtained, it was found that the robustness parameters were within the limits at all variable conditions.

LOD and LOQ

The detection limit (LOD) of an individual analytical procedure is the lowest amount of analyte in a sample, which can be detected but not necessarily quantified as an exact value. The quantification limit (LOQ) is a parameter of quantitative assay for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products. The LOD and LOQ can be found based on

1. Visual Evaluation
2. Signal-to-Noise ratio
3. The standard deviation of the response and the slope.

In the present investigation, LOD and LOQ were determined from standard deviation and slope methods as per ICH guidelines and calculated from the average of slope and standard deviation (n=3) of the intercepts of the regression equation. LOD for Minocycline hydrochloride was found to be 0.6065µg/mL LOQ for Minocycline hydrochloride was found to be 1.838µg/mL, indicating good sensitivity of the method.

System suitability

System suitability testing is an integral part of any analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations, and samples to be analyzed constitute an integral system factor are parameters that are normally used in assessing the column performance.

System suitability studies were carried out by injecting five times standard concentration of 10µg/mL (Minocycline hydrochloride) at different injection volumes ranging from 10µL to 50µL. The RSD values for system suitability test

parameters like retention time[R_t = 1.2560 for Minocycline hydrochloride], tailing factor [T_f = 1.2018 for Minocycline hydrochloride] and theoretical plate number [0.449 for Minocycline hydrochloride] were found to be less than 2% indicating the present conditions were suitable for the analysis of Minocycline hydrochloride in tablets. The data was given in Table 3.6 and Figures 3.40-3.45.

Acceptance Criteria

- Theoretical plate should be N>2000
- Tailing factor should be T<2

Table 3.6 System suitability data for Minocycline hydrochloride

InjectionVol. (µL)	Retention time (min)	Tailing factor (T_f)	Theoretical plate (#)
10	4.175	1.426	2441.07
20	4.212	1.381	2452.19
30	4.244	1.471	2459.28
40	4.279	1.408	2471.39
50	4.310	1.412	2457.79
Mean	4.244	1.4088	2456.346
% RSD	1.2560	1.2018	0.449

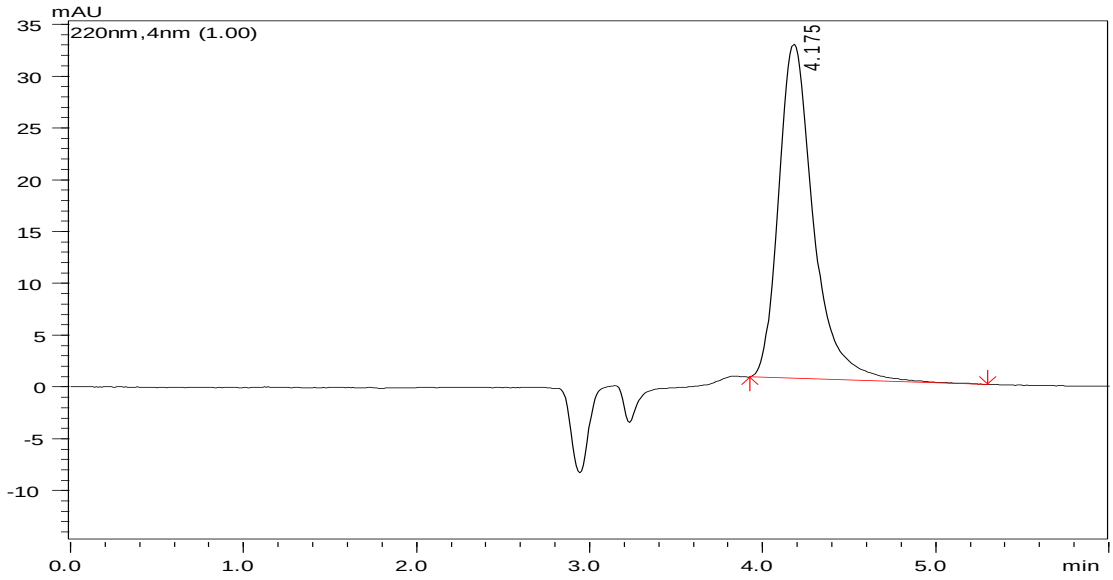


Fig. 3.40 System suitability chromatogram of standard solution minocycline hydrochloride-10µL

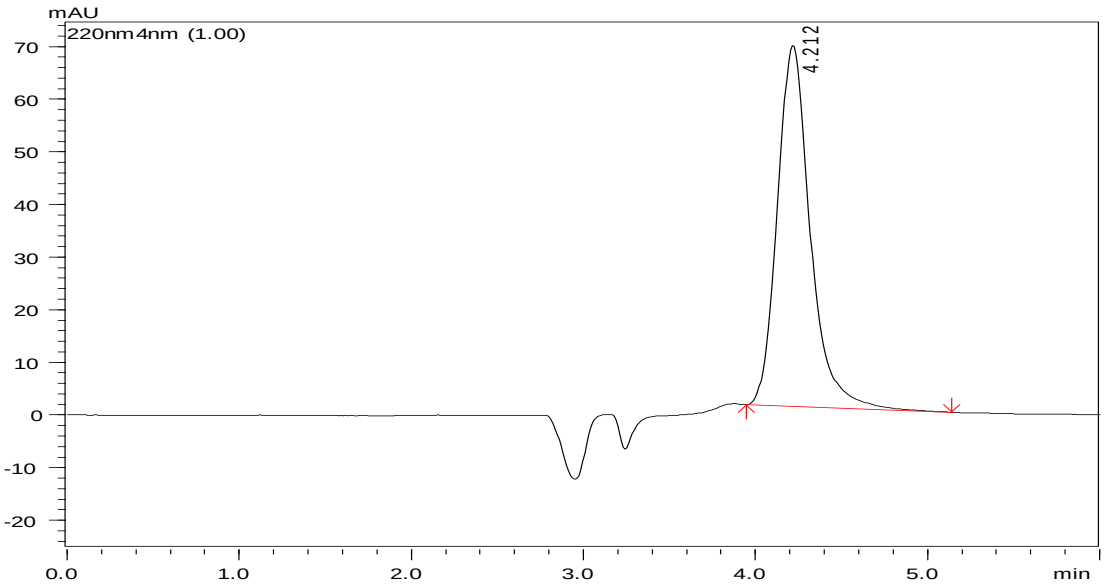


Fig. 3.41 System suitability chromatogram of standard solution of minocycline hydrochloride-20µL

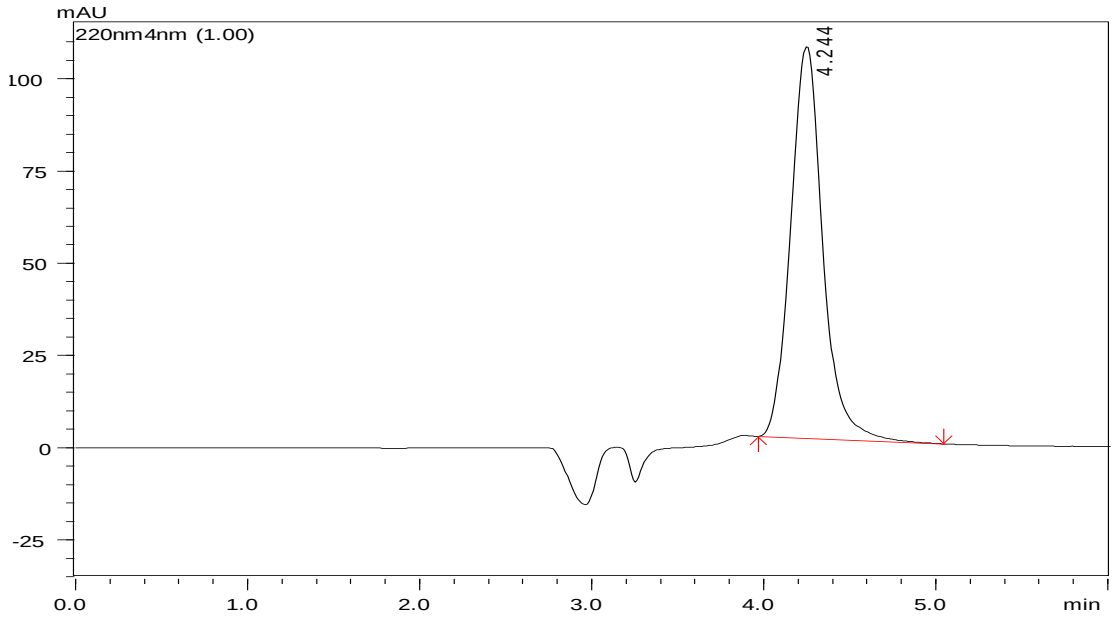


Fig. 3.42 System suitability chromatogram of standard solution of minocycline hydrochloride-30µL

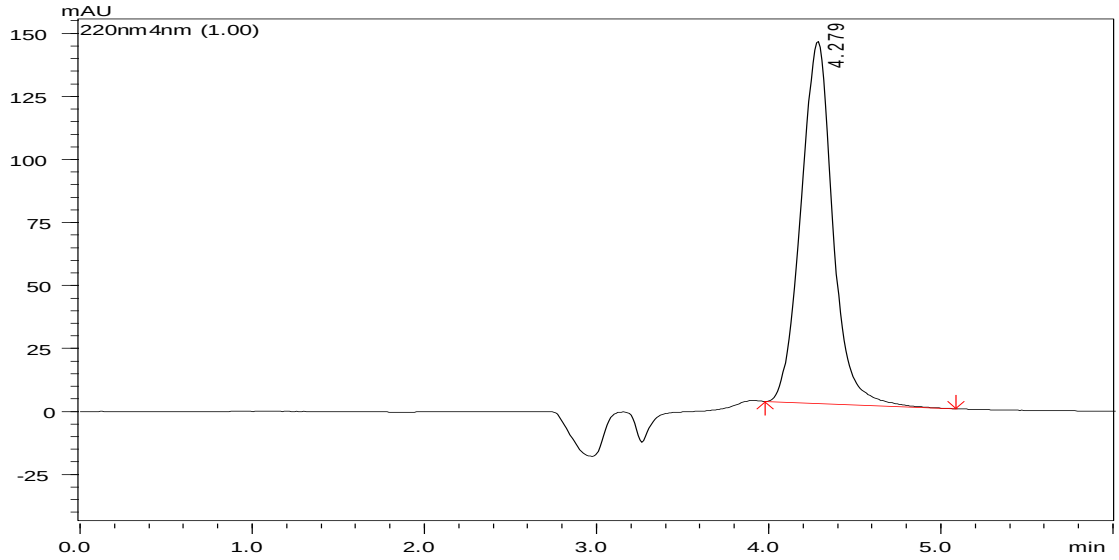


Fig. 3.43 System suitability chromatogram of standard solution of minocycline hydrochloride-40µL

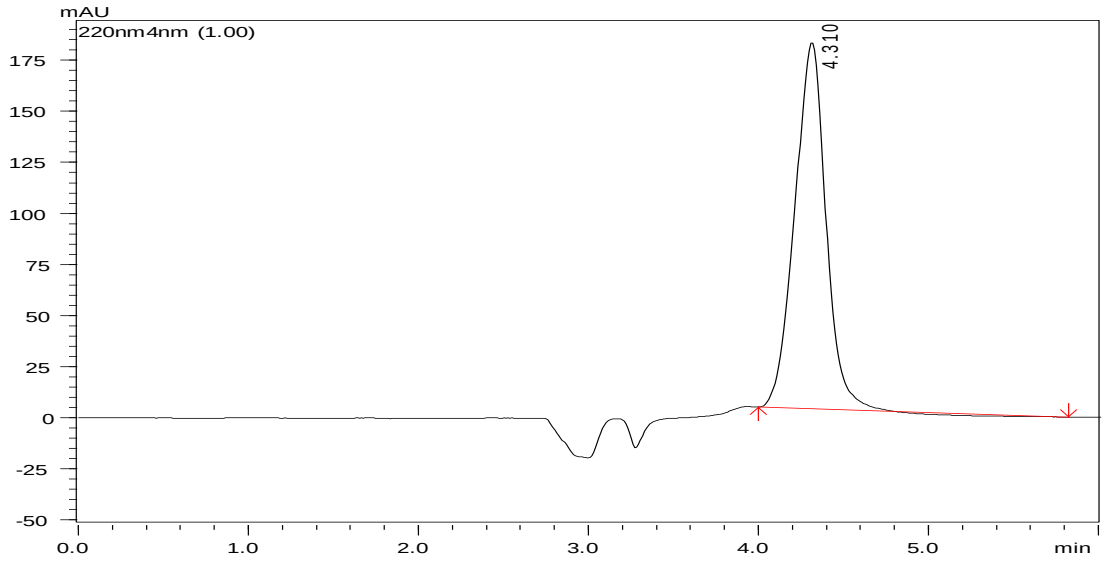
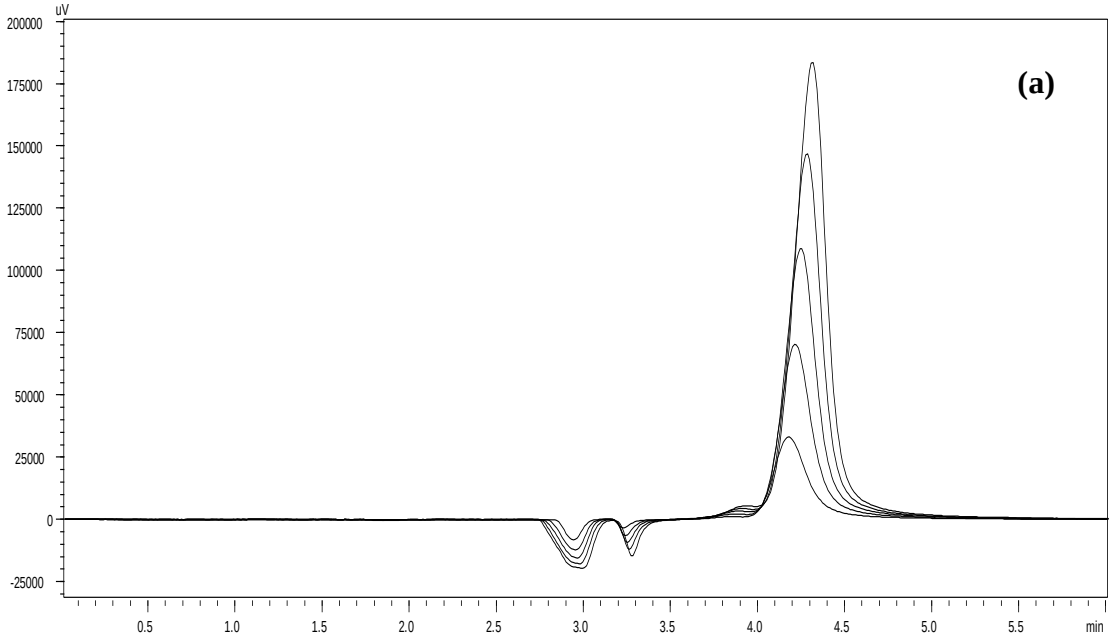


Fig. 3.44 System suitability chromatogram of standard solution of minocycline hydrochloride-50µL



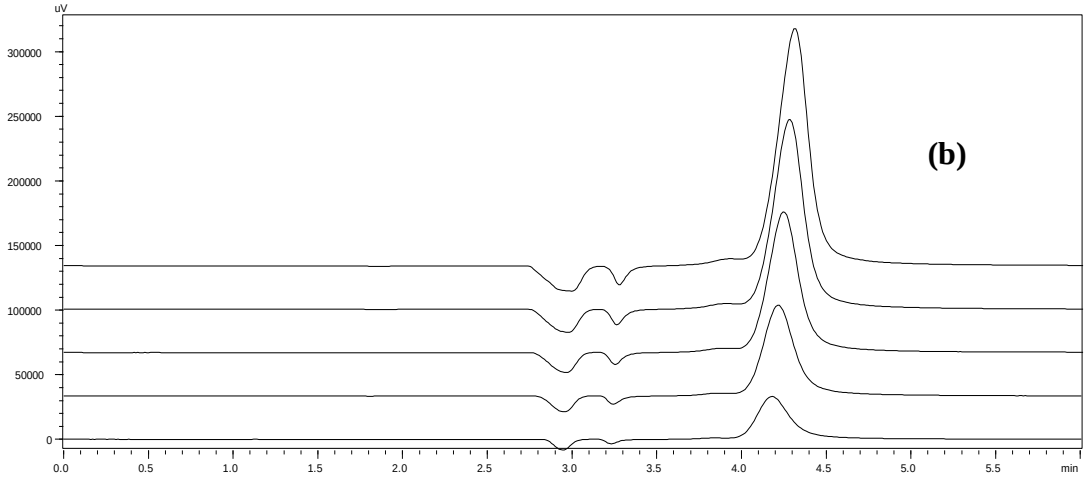


Fig. 3.45 Overlay {(a) without base shift (b) with base shift} of System suitability chromatograms of standard solution (10-50µL) of minocycline hydrochloride.

Observation

From the observation it was found that the system suitability test parameters were within limits for the proposed method.

Stability of the Stock Solution

The stability of the stock and standard solutions were determined by analyzing the samples under refrigeration (8±1˚C) at different time intervals up to 24 hours. The % variation in assay values at different time intervals were found to be less than 2 of the initial zero time interval solution, thus indicating that the solutions were stable for a period of 24 hours when stored at 8ºC. The data was given in Table 3.7 and shown in Figures 3.93-3.94.

Table 3.7 Stability data for minocycline hydrochloride

Time interval	%Variation in peak area
	Minocycline hydrochloride
0 hrs	446782
24 hrs	443187

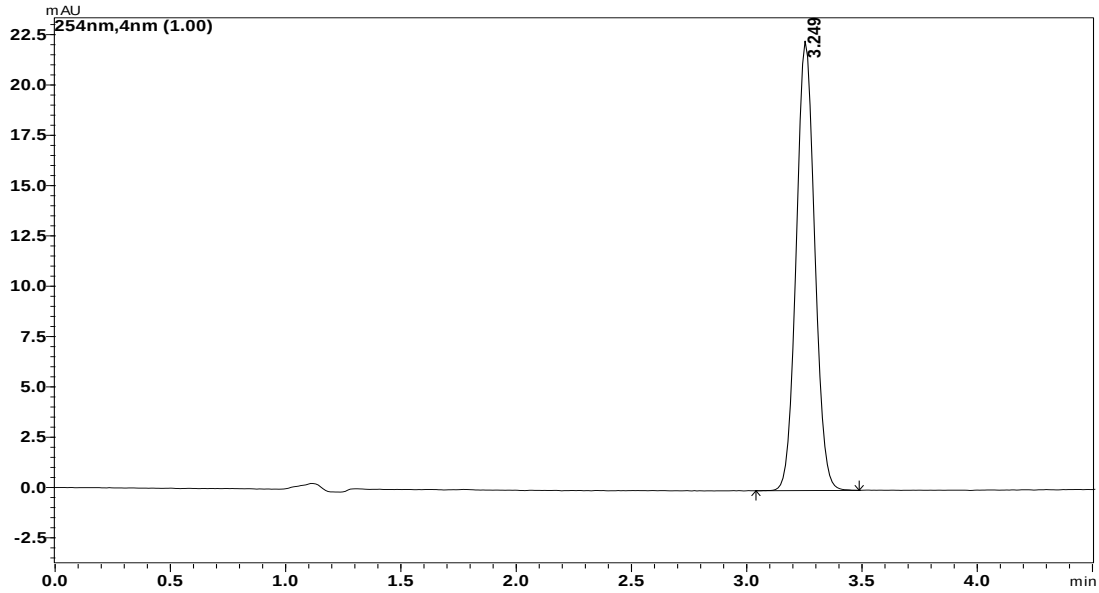


Fig. 3.46 Stability chromatogram of minocycline hydrochloride at 0 hr (30 µg/mL of minocycline hydrochloride)

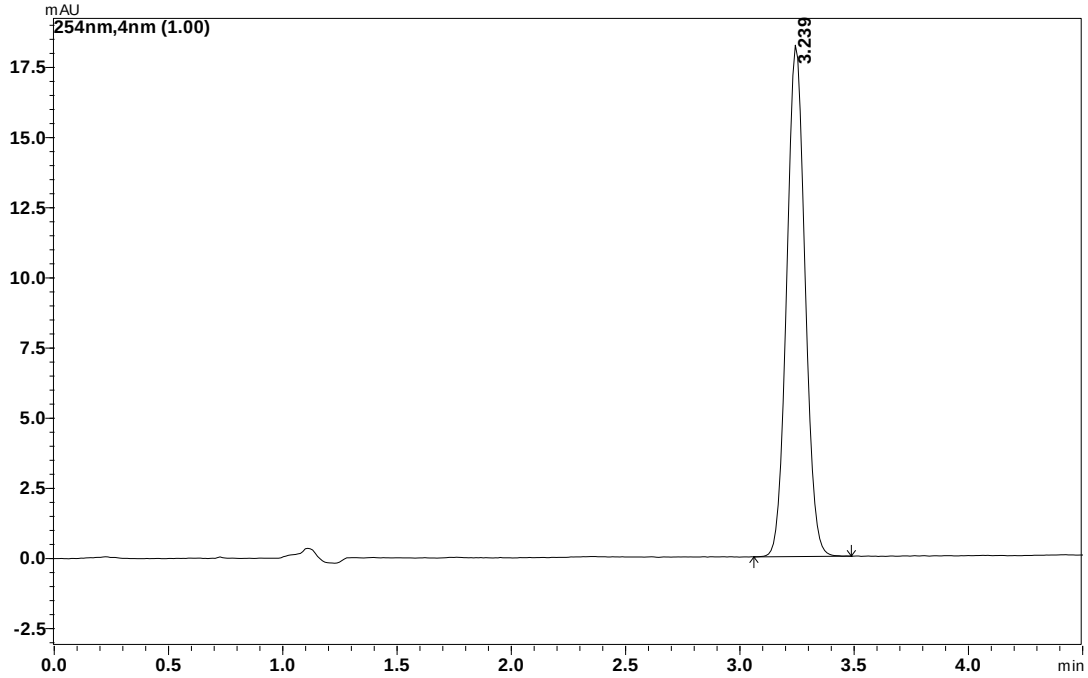


Fig. 3.47 Stability chromatogram of minocycline hydrochloride at 24 hrs (30µg/mL of minocycline hydrochloride)

Observation

From the results obtained, it was found that the %variation is less than 2.

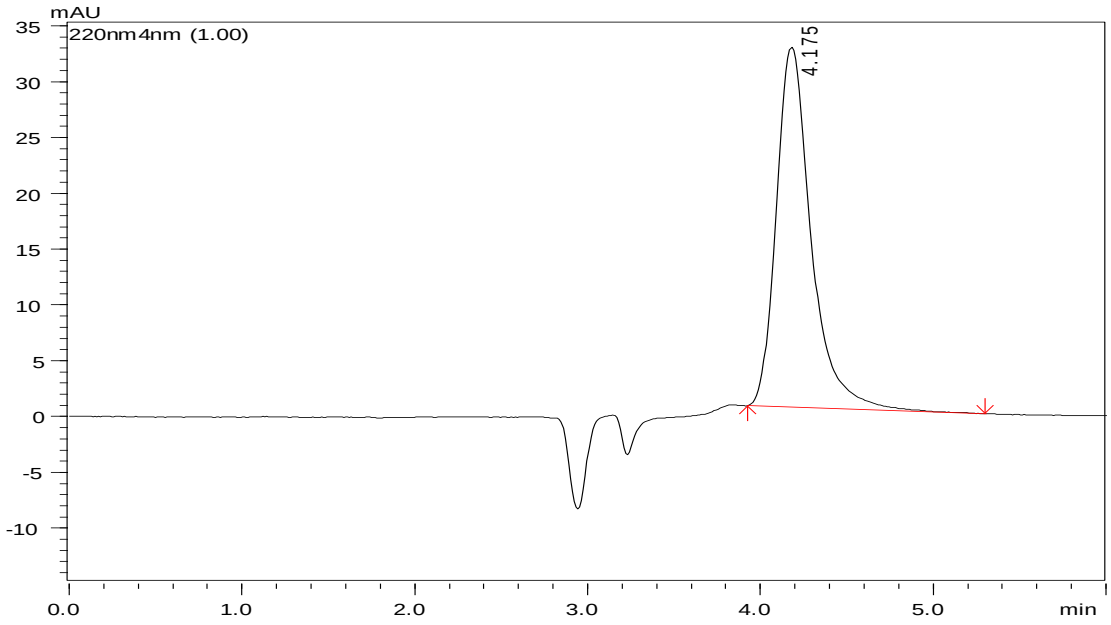


Fig. 3.48 Assay chromatogram of formulation minocycline hydrochloride

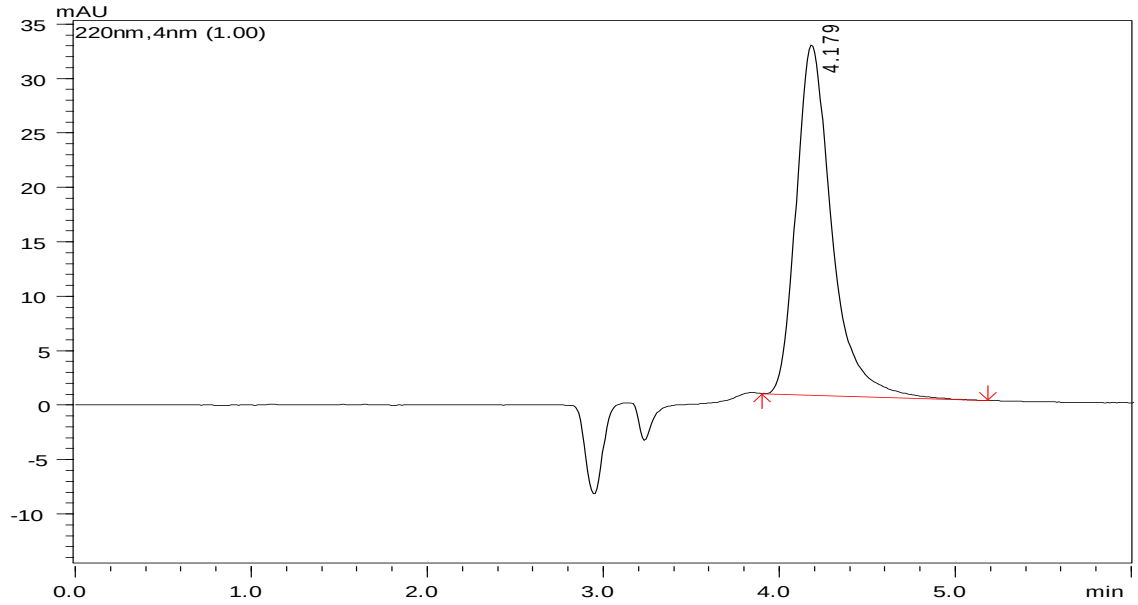


Fig. 3.49 Assay chromatogram of formulation minocycline hydrochloride

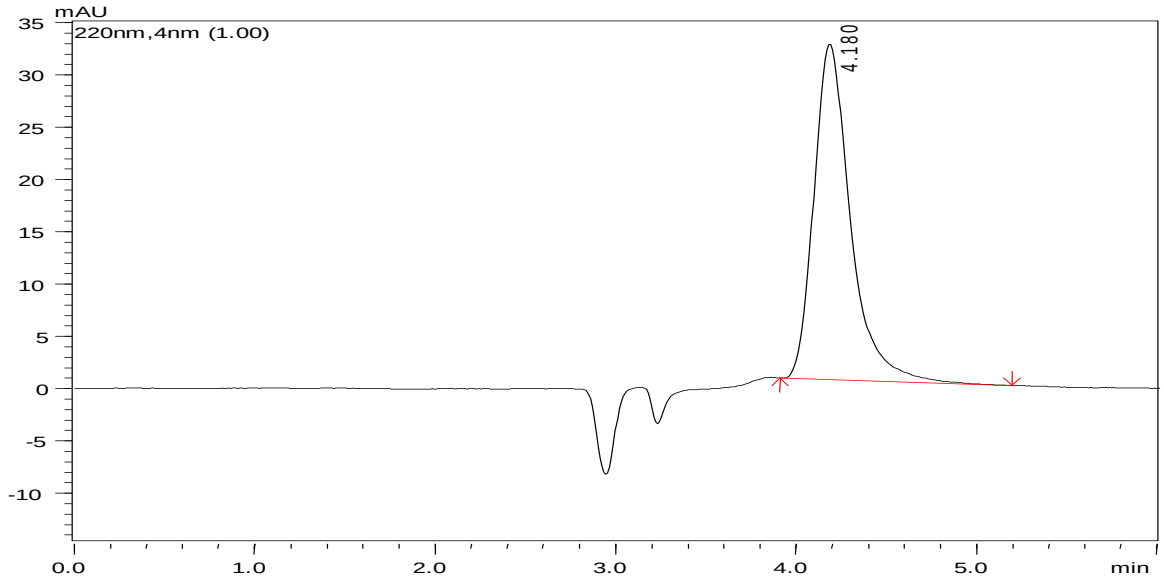


Fig. 3.50 Assay chromatogram of formulation minocycline hydrochloride

Observation

The percentage RSD value of assay for minocycline hydrochloride was within the limits.

7. SUMMARY AND CONCLUSIONS

Minocycline hydrochloride is a broad-spectrum tetracycline antibiotic, chemically it is (4S,4aS,5aR,12aR)-4,7-bis(dimethylamino)-1,10,11,12a-tetrahydroxy-3,12-dioxo-3,4,4a,5,5a,6, 12,12a-octahydrotetracene-2-carboximidic acid hydrochloride. Minocycline passes directly through the lipid bilayer or passively diffuses through porin channels in the bacterial membrane. Tetracyclines like minocycline bind to the 30S ribosomal subunit, preventing the binding of tRNA to the mRNA-ribosome complex and interfering with protein synthesis. The aim of this present investigation is to develop an efficient, rapid and sensitive RP-HPLC-PDA method for the estimation of Minocycline hydrochloride in bulk, pharmaceutical dosage forms compatible for LC-MS methods and subsequent validation of the LC method as per ICH guidelines.

METHOD DEVELOPMENT

Finally these are the optimized conditions developed after performing suitable trials.

Mobile phase	:	0.02%V/v/v Formic acid:Methanol (70:30v/v)
Flow rate	:	1.0 mL/min
Column	:	Inertsil ODS Column (150x4.6 mm, 5µ)
Type of elution	:	Isocratic
Detector wave length	:	220 nm
Column temperature	:	Ambient
Pressure observed	:	62 kgf
Injection volume	:	10µL
Run time	:	7 min

With the optimized conditions mentioned above, peak was eluted at 4.17 min, with good peak symmetry and with acceptable results of system suitability parameters.

METHOD VALIDATION

Specificity

The specificity of the method was established by spiking solution of commonly used excipients. Peak purity tests were also carried out to show that the analyte chromatographic peak is not attributable to more than one component as the impurities are not available by purity index data, as there was no interference of impurities with the analyte peak. This shows that the peak of analyte was pure and excipients in the formulation did not interfere with the analyte.

- From the chromatograms and 3 D plots it can be inferred that there were no co eluting or interfering peaks where minocycline hydrochloride was eluted and the peak purity value greater than 0.9999 for minocycline hydrochloride this showed that the method was specific.

Linearity

Linearity studies were performed at 10-50µg/mL of Standard drug solution of minocycline hydrochloride by constructing graph between concentrations vs. peak areas. % RSD of peak areas were determined. Correlation coefficient was calculated, the data found within the acceptance criteria of 0.99.

- The result was found to be within limit so the method is linear over the concentration range of 10-50µg/mL of Minocycline hydrochloride.

Precision

Precision of related substances were verified by repeatability. Repeatability was assessed by using a minimum of six determinations at 100 % of the test concentration (30µg/mL of minocycline hydrochloride) standard deviation and relative standard deviation were reported for precision.

- % RSD of area response of minocycline hydrochloride was 0.152 for system precision and 0.317 for method precision

- % RSD of retention times of minocycline hydrochloride was 0.055 for system precision and 0.061 for method precision

- % RSD should be NMT 2 for areas and NMT 1 for retention times. It indicates that the method is precise.

Accuracy

Accuracy of the proposed method was ascertained by performing recovery studies by standard addition method by spiking the known quantities of standard at 80%, 100%, 120% to the drug product solution comprising of 30µg/mL. The % RSD and the % Recovery were within the acceptable limits of 98-102 in all cases.

- The recovery of minocycline hydrochloride was found to be 99.52-100.66%, which indicates a good accuracy of the method to that of the label claim.

LOD &LOQ

The quantification limit of individual analytical procedure is the lowest amount of analyte in the sample that can be determined quantitatively. LOD and LOQ were calculated from the average slope and standard deviation from the calibration curve.

- LOD for minocycline hydrochloride was found to be 0.6065µg/mL, indicating high sensitivity of the method.

- LOQ for minocycline hydrochloride was found to be 1.838µg/mL, indicating high sensitivity of the method.

Robustness

As part of the robustness, deliberate changes in the flow rate and wavelength were made to evaluate the impact on the method.

- The obtained results indicated that the minor changes in the flow rate and wave length did not affect the actual conditions.

System suitability

System suitability was used to verify the reproducibility of the chromatographic system. To ascertain its effectiveness, system suitability were carried out on freshly prepared stock solution. System suitability parameters are evaluated by measuring the closeness of the obtained values % RSD for the parameters like tailing factor of minocycline hydrochloride was found to be 1.20, retention time of minocycline hydrochloride was found to be 1.25 and the theoretical plates of minocycline hydrochloride was found to be 0.449

- The % RSD of tailing factor, retention time and theoretical plates of minocycline hydrochloride was found to be less than 2.

Stability of the solution

The stability of the solutions was assessed by analysing the sample and standard solution at different time intervals up to 24 hours and the % variation of minocycline hydrochloride was found to be 0.293%. It was found out that the solution remained stable for 24 hours when stored at 8°C.

- The Final value should be within 2% of the initial value.

Assay

The assay of Minocycline hydrochloride tablets was performed by comparing the areas of standard minocycline hydrochloride and tablet sample,

- The percentage assay for minocycline hydrochloride was found to be 99.43%.
- Assay was performed by the data was found suitable and within the acceptance range of 100 ± 2 %.

The Table 4.1 below summarizes the results of various parameters of validation comparing the obtained results with the acceptance criteria.

Table 4.1 Summary of results of Validation Parameters

Parameter		Acceptance Criteria	Result
Specificity		No interference from blank, placebo and other degradation products with the main peak.	No interference from blank, placebo and other degradation products with the main peak.
		Peak purity index > 0.999	Peak purity index =0.9999 for Minocycline hydrochloride
Linearity		$R^2 > 0.99$	$R^2 = 0.999$ for Minocycline hydrochloride
Precision	System	RSD of area < 2%	RSD of area for Minocycline hydrochloride = 0.152
	Method	RSD of area < 2%	RSD of area for Minocycline hydrochloride = 0.317
Accuracy		98- 102%	99.52-100.66 for Minocycline hydrochloride
LOD		S/N > 2 or 3	0.6065µg/mL for Minocycline hydrochloride
LOQ		S/N > 10	1.838µg/mL for Minocycline hydrochloride
Robustness		No significant affect	Slight variations in retention times
System Suitability		Tailing factor < 2	Tailing factor for Minocycline hydrochloride = 1.4088
		Theoretical plate number > 2000	Theoretical plate number for Minocycline hydrochloride = 2456.346.
Solution stability		% variation< 2 for 24 hrs	%variation of Minocycline hydrochloride = 0.293%
Assay		98-100%	99.43% for Minocycline hydrochloride

Finally, it can be concluded that the proposed RP-HPLC-PDA method was validated fully as per the International Conference on Harmonization (ICH) Guidelines, and found to be applicable for routine quality control analysis for the estimation of Minocycline hydrochloride. The results of linearity, precision, accuracy and specificity proved to be within the limits. The method provides selective quantification of Minocycline hydrochloride without interference from blank, placebo and degradants. The proposed method is sensitive, reproducible, reliable, rapid, economical, and specific and LC-MS compatible, can be used for the estimation of Minocycline hydrochloride.

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